Gene Expression DEG Practical

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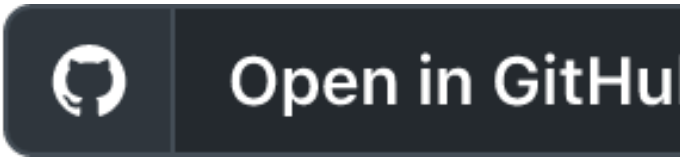
Gene Expression DEG practical

Start Codespaces to run this practical on the cloud! 

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Máster en Métodos Computacionales en Ciencias - Análisis e interpretación de datos de alto rendimiento. UNAV

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Introduction

Students will be handed real human RNA-seq data (Himes et al 2014) to investigate differential gene expression (DGE) under different experimental conditions.

Characterization transcriptomic changes in four primary human ASM cell lines that were treated with dexamethasone — a potent synthetic glucocorticoid (1 /μM for 18 hours). R and Bioconductor statistical packages to research variation at gene level. R studio NGS visualization technologies will be used to display gene expression differences and gene ontology to infer biological meaning of their findings.

Additional Reading and Credits

This practice material was adapted from Michael Love, DeSeq2 tutorial 2018 (kindly authorized us to reproduce). RNA-seq workflow: gene-level exploratory analysis and differential expression. 2018 **

https://www.bioconductor.org/packages/devel/workflows/vignettes/rnaseqGene/inst/doc/rnaseqGene.html

Introduction to DGE. Teaching materials at the Harvard Chan Bioinformatics Core *These are open access materials distributed under the terms of the Creative Commons Attribution license (CC BY 4.0), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.* https://hbctraining.github.io/DGE_workshop/lessons/04_DGE_DESeq2_analysis.html

Introduction to differential gene expression analysis using RNA-seq. Friederike Dündar, Luce Skrabanek, Paul Zumbo 2015 * http://chagall.med.cornell.edu/RNASEQcourse/Intro2RNAseq.pdf

Interesting paper describing all the steps in a RNA-seq DGE experiment ** Conesa A, Madrigal P, Tarazona S, et al. A survey of best practices for RNA-seq data analysis. Genome Biology. 2016;17:13. doi:10.1186/s13059-016-0881-8.

Love, Michael I., et al. "RNA-Seq workflow: gene-level exploratory analysis and differential expression." F1000Research 4 (2015). *

List of RNA-Seq bioinformatics tools

Extensive list of RNA-seq bioinformatic tools.

https://en.wikipedia.org/wiki/List_of_RNA-Seq_bioinformatics_tools

Differential gene expression using R and Bioconductor packages.

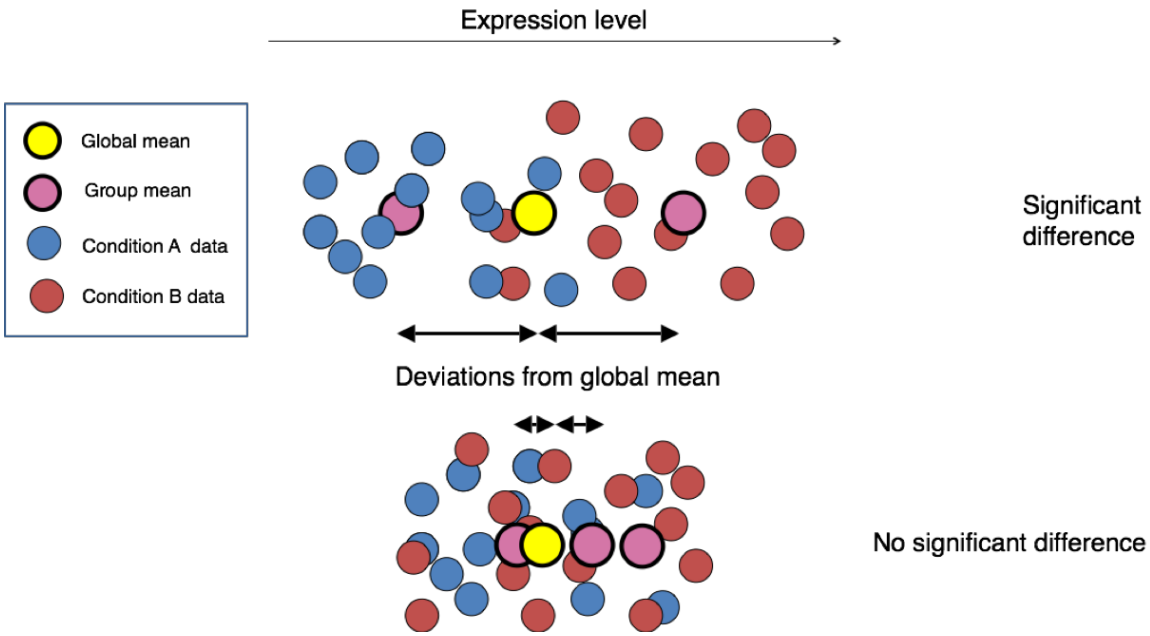


Figure 1: mean normalization

DGE bioinformatic schema

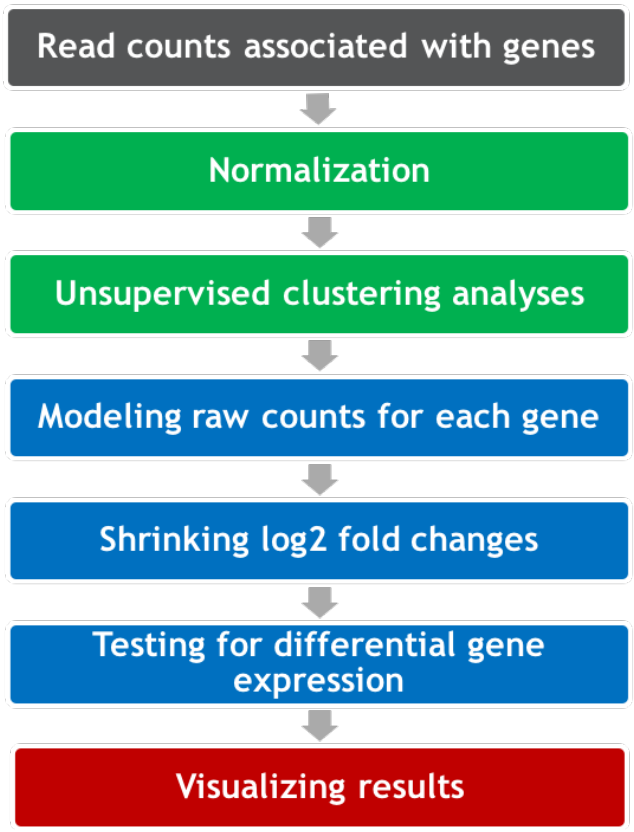


Figure 2: DGE bioinformatic schema

Airway data load

```
# # Download BiocManager
# if (!requireNamespace("BiocManager", quietly = TRUE))
#   install.packages("BiocManager")
# # Download airway library
# BiocManager::install("airway")


## For old R distribution <4.0
# source("https://bioconductor.org/biocLite.R")
# #biocLite("airway")
# if (!require(airway)) {
#     install.packages("airway")
#     require(airway)
# }
```

```r
# Load airway
library("airway")

# Install and load SummarizedExperiment
# BiocManager::install("SummarizedExperiment")
library("SummarizedExperiment")

# Check airway vignette
# vignette("airway")

# Load airway data
data("airway")


# Assign airway data to summarized experiment R object
se <- airway


# Set directory
# WARNIG, change to your system path!!!

# Load directory to Knit
knitr::opts_knit$set(root.dir = "/workspaces/codespaces_RStudio/course_materials/RNA_seq_DEG"

# # Load directory from MAC
# directory <-"/Users/mozosi/Dropbox (UCL-MN Team)/Class/Unav2021/ML/DGE"

# Load directory from Windows
# directory <- "C:/Users/d682011.ADMON-CFNAVARRA/OneDrive - NAVARRA SOCIEDADES PÚBLICAS/Other
directory <- "/workspaces/codespaces_RStudio/course_materials/RNA_seq_DEG"

setwd(directory)
list.files()
```

```
[1] "DESeq2_Sig_results_dex_Vs_untrt.tab"
[2] "in.txt"
[3] "Merge_GO_Gene_Set_Enrichment_Analysis_CC_.pdf"
[4] "se_data.rds"
[5] "Significant_DE_dex_trt_vs_untrt_metadata.tab"
```

```r
# Save an object to a file
saveRDS(se, file = "se_data.rds")

# Restore the object. In case you will need to restore the data object
# readRDS(file = "se_data.rds")
```

Summarized experiment.

The component parts of a SummarizedExperiment object. The assay (pink block) contains the matrix of counts, the rowRanges (blue block) contains information about the genomic ranges and the colData (green block) contains information about the samples. The highlighted line in each block represents the first row (note that the first row of colData lines up with the first column of the assay).

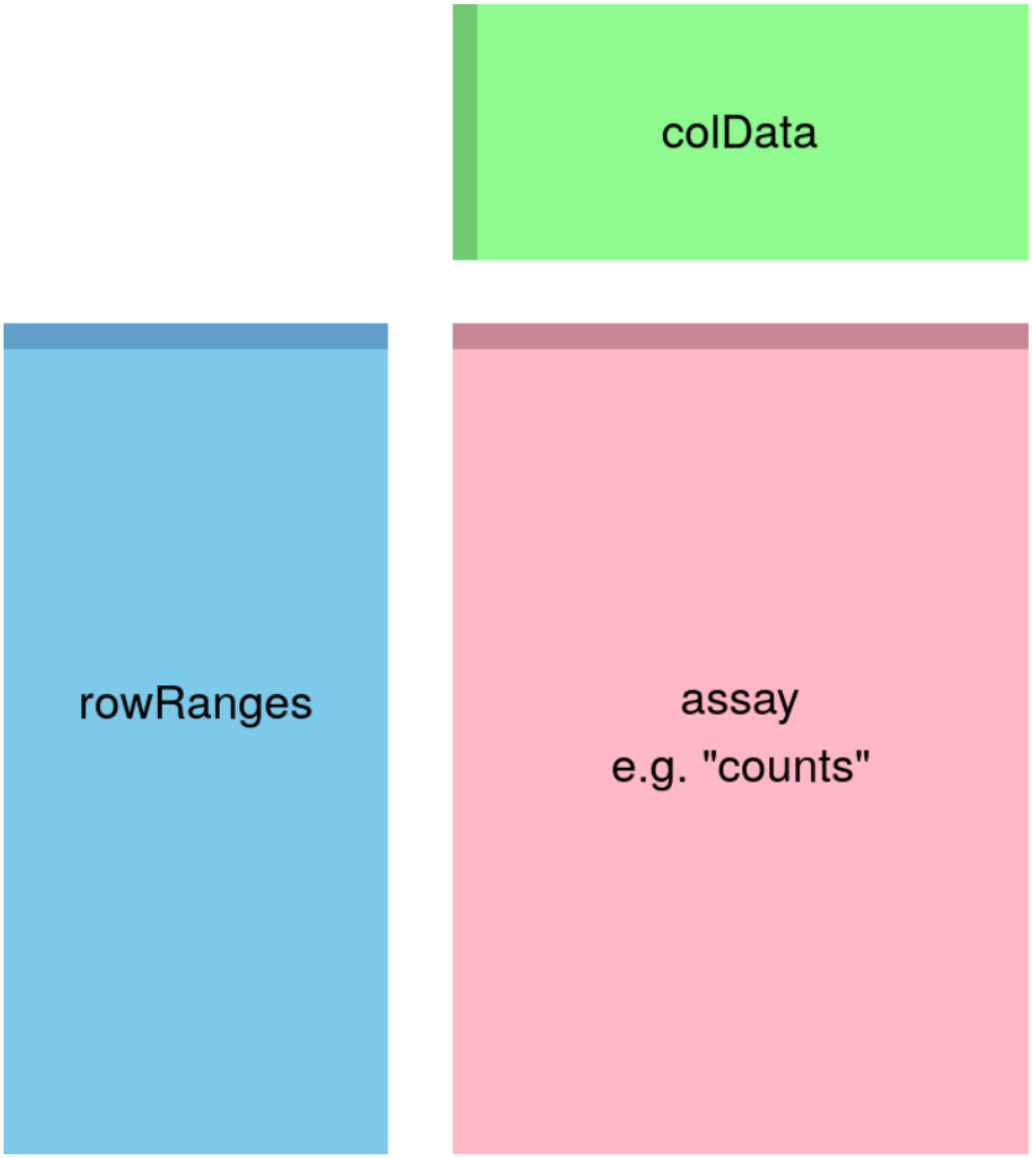


Figure 3: Summarized experiment structure

```
# Total sum of read counts
colSums(assay(se))
```

```
SRR1039508 SRR1039509 SRR1039512 SRR1039513 SRR1039516 SRR1039517 SRR1039520
  20637971   18809481   25348649   15163415   24448408   30818215   19126151
SRR1039521
```

```
  21164133
```

```
# Head of summarizeexperiment object
head(assay(se), 3)
```

```
                SRR1039508 SRR1039509 SRR1039512 SRR1039513 SRR1039516
ENSG00000000003        679        448        873        408       1138
ENSG00000000005          0          0          0          0          0
ENSG00000000419        467        515        621        365        587
                SRR1039517 SRR1039520 SRR1039521
ENSG00000000003       1047        770        572
ENSG00000000005          0          0          0
ENSG00000000419        799        417        508
```

```
# Gene genomic ranges with genomic ranges
rowRanges(se)
```

```
GRangesList object of length 63677:
$ENSG00000000003
GRanges object with 17 ranges and 2 metadata columns:
       seqnames            ranges strand |   exon_id       exon_name
          <Rle>         <IRanges>  <Rle> | <integer>     <character>
   [1]        X 99883667-99884983      - |    667145 ENSE00001459322
   [2]        X 99885756-99885863      - |    667146 ENSE00000868868
   [3]        X 99887482-99887565      - |    667147 ENSE00000401072
   [4]        X 99887538-99887565      - |    667148 ENSE00001849132
   [5]        X 99888402-99888536      - |    667149 ENSE00003554016
   ...      ...               ...    ... .       ...             ...
  [13]        X 99890555-99890743      - |    667156 ENSE00003512331
  [14]        X 99891188-99891686      - |    667158 ENSE00001886883
  [15]        X 99891605-99891803      - |    667159 ENSE00001855382
  [16]        X 99891790-99892101      - |    667160 ENSE00001863395
  [17]        X 99894942-99894988      - |    667161 ENSE00001828996
  -------
  seqinfo: 722 sequences (1 circular) from an unspecified genome

...
<63676 more elements>
```

```
# Metada contained in the ranges
str(metadata(rowRanges(se)))
```

```
List of 1
 $ genomeInfo:List of 20
  ..$ Db type                                    : chr "TranscriptDb"
  ..$ Supporting package                         : chr "GenomicFeatures"
  ..$ Data source                                : chr "BioMart"
  ..$ Organism                                   : chr "Homo sapiens"
  ..$ Resource URL                               : chr "www.biomart.org:80"
  ..$ BioMart database                           : chr "ensembl"
  ..$ BioMart database version                   : chr "ENSEMBL GENES 75 (SANGER UK)"
  ..$ BioMart dataset                            : chr "hsapiens_gene_ensembl"
  ..$ BioMart dataset description                : chr "Homo sapiens genes (GRCh37.p13)"
  ..$ BioMart dataset version                    : chr "GRCh37.p13"
  ..$ Full dataset                               : chr "yes"
  ..$ miRBase build ID                           : chr NA
  ..$ transcript_nrow                            : chr "215647"
  ..$ exon_nrow                                  : chr "745593"
  ..$ cds_nrow                                   : chr "537555"
  ..$ Db created by                              : chr "GenomicFeatures package from Bioconducto
  ..$ Creation time                              : chr "2014-07-10 14:55:55 -
0400 (Thu, 10 Jul 2014)"
  ..$ GenomicFeatures version at creation time: chr "1.17.9"
  ..$ RSQLite version at creation time           : chr "0.11.4"
  ..$ DBSCHEMAVERSION                            : chr "1.0"
```

```
# Annotation of the experiment
colData(se)
```

```
DataFrame with 8 rows and 9 columns
           SampleName     cell      dex    albut        Run avgLength
             <factor> <factor> <factor> <factor>   <factor> <integer>
SRR1039508 GSM1275862  N61311     untrt    untrt SRR1039508       126
SRR1039509 GSM1275863  N61311     trt      untrt SRR1039509       126
SRR1039512 GSM1275866  N052611    untrt    untrt SRR1039512       126
SRR1039513 GSM1275867  N052611    trt      untrt SRR1039513        87
SRR1039516 GSM1275870  N080611    untrt    untrt SRR1039516       120
SRR1039517 GSM1275871  N080611    trt      untrt SRR1039517       126
SRR1039520 GSM1275874  N061011    untrt    untrt SRR1039520       101
SRR1039521 GSM1275875  N061011    trt      untrt SRR1039521        98
```

```
           Experiment    Sample    BioSample
             <factor>  <factor>     <factor>
SRR1039508  SRX384345 SRS508568 SAMN02422669
SRR1039509  SRX384346 SRS508567 SAMN02422675
SRR1039512  SRX384349 SRS508571 SAMN02422678
SRR1039513  SRX384350 SRS508572 SAMN02422670
SRR1039516  SRX384353 SRS508575 SAMN02422682
SRR1039517  SRX384354 SRS508576 SAMN02422673
SRR1039520  SRX384357 SRS508579 SAMN02422683
SRR1039521  SRX384358 SRS508580 SAMN02422677
```

```
# Check object attributes and class
#attributes(se)
class(se)
```

```
[1] "RangedSummarizedExperiment"
attr(,"package")
[1] "SummarizedExperiment"
```

Differential gene expression with DeSeq2

DeSeq2 is one of the most widely used DGE packages (Love 2014).
* Don't assume that variance and median are equal – not normally distributed. * Allow global variance and check the variance of a single gene versus the rest and whether the changes are due to chance. * In essence, we are testing if the mean expression are significantly different. The null hypothesis is that there is no systematic difference between the average read count values of the different conditions for a given gene.

```
# Download DeSeq2 if necesary
# if (!require(DESeq2)) {
#     install.packages("DESeq2")
#     require(DESeq2)
# }
# BiocManager::install("DESeq2")
# BiocManager::install("DelayedArray")
```

The DESeq2 model

The DESeq2 model and all the steps taken in the software are described in detail in our publication (Love, Huber, and Anders 2014), and we include the formula and descriptions in

this section as well. The differential expression analysis in DESeq2 uses a generalized linear model of the form:

Kij␣"~"␣NB(/\)\mu\(ij,/\)\alpha\(i)∗

/\)\mu\(ij=sjqij∗

log2(qij)=xj./\)\beta\(i∗

where counts **Kij** for gene i, sample j are modeled using a negative binomial distribution with fitted mean /μij and a gene-specific dispersion parameter /αi. The fitted mean is composed of a sample-specific size factor sj and a parameter qij proportional to the expected true concentration of fragments for sample j. The coefficients /βi give the log2 fold changes for gene i for each column of the model matrix X. Note that the model can be generalized to use sample- and gene-dependent normalization factors sij

.

The dispersion parameter */αi* defines the relationship between the variance of the observed count and its mean value. In other words, how far do we expected the observed count will be from the mean value, which depends both on the size factor sj and the covariate-dependent part *qij* as defined above.

Var(Kij)=E[(Kij-/\)\mu\(ij)2]=/\)\mu\(ij+/\)\alpha\(i/\)\mu\(2ij∗

An option in DESeq2 is to provide maximum a posteriori estimates of the log2 fold changes in */βi* after incorporating a zero-centered Normal prior (betaPrior). While previously, these moderated, or shrunken, estimates were generated by DESeq or nbinomWaldTest functions, they are now produced by the lfcShrink function. Dispersions are estimated using expected mean values from the maximum likelihood estimate of log2 fold changes, and optimizing the Cox-Reid adjusted profile likelihood, as first implemented for RNA-seq data in edgeR (Cox and Reid 1987,edgeR_GLM). The steps performed by the DESeq function are documented in its manual page DESeq; briefly, they are:

1. estimation of size factors sj by *estimateSizeFactors*
2. estimation of dispersion /αi by *estimateDispersions*
3. negative binomial GLM fitting for /βi and Wald statistics by *nbinomWaldTest*

For access to all the values calculated during these steps, see the link below

http://bioconductor.org/packages/devel/bioc/vignettes/DESeq2/inst/doc/DESeq2.html#access

```
# Load DeSeq2
library("DESeq2")

# Order of factors before relevel
se$dex
```

```
[1] untrt trt   untrt trt   untrt trt   untrt trt
Levels: trt untrt
```

```
# When we talk about differentially gene expression (DGE) we are looking for changes in expre
# the treatment Vs control. Relevel untreated samples (Note: it is preferred in R that the fi
# of a factor be the reference level (e.g. control, or untreated samples), so we can relevel
# factor. DeSeq2 will consider the first level as control and will perform pair-wise comparis
# first factor is the denominator on the fold change variation.

se$dex <- relevel(se$dex, "untrt")
# Order of factors after relevel. Untreated came to the first position on the factors levels
se$dex
```

```
[1] untrt trt   untrt trt   untrt trt   untrt trt
Levels: untrt trt
```

```
# Construct a DESeqDataSet data object
dds_raw <- DESeqDataSet(se, design = ~ cell + dex)
```

Note on sample comparision

DO NOT RUN! contrast <- c("condition", "treatment", "control") results(dds, contrast = contrast, alpha = alpha_threshold)

 # Result table should have the control at the end of the comparison log2 fold change (MAP): sampletype treatment vs control Wald test p-value: sampletype MOV10_overexpression vs control DataFrame with 6 rows and 6 columns baseMean log2FoldChange lfcSE stat pvalue padj

Check Individual gene plot to asses your analysis!!

```
# In essence we have the same data but now is a DeSeq2 data object
colData(dds_raw)
```

```
DataFrame with 8 rows and 9 columns
           SampleName     cell      dex    albut        Run avgLength
             <factor> <factor> <factor> <factor>   <factor> <integer>
SRR1039508 GSM1275862  N61311     untrt    untrt SRR1039508       126
SRR1039509 GSM1275863  N61311     trt      untrt SRR1039509       126
SRR1039512 GSM1275866  N052611    untrt    untrt SRR1039512       126
SRR1039513 GSM1275867  N052611    trt      untrt SRR1039513        87
```

```
SRR1039516 GSM1275870  N080611    untrt    untrt SRR1039516       120
SRR1039517 GSM1275871  N080611    trt      untrt SRR1039517       126
SRR1039520 GSM1275874  N061011    untrt    untrt SRR1039520       101
SRR1039521 GSM1275875  N061011    trt      untrt SRR1039521        98
           Experiment    Sample    BioSample
             <factor>  <factor>     <factor>
SRR1039508  SRX384345 SRS508568 SAMN02422669
SRR1039509  SRX384346 SRS508567 SAMN02422675
SRR1039512  SRX384349 SRS508571 SAMN02422678
SRR1039513  SRX384350 SRS508572 SAMN02422670
SRR1039516  SRX384353 SRS508575 SAMN02422682
SRR1039517  SRX384354 SRS508576 SAMN02422673
SRR1039520  SRX384357 SRS508579 SAMN02422683
SRR1039521  SRX384358 SRS508580 SAMN02422677
```

```
head(assay(dds_raw))
```

```
                SRR1039508 SRR1039509 SRR1039512 SRR1039513 SRR1039516
ENSG00000000003        679        448        873        408       1138
ENSG00000000005          0          0          0          0          0
ENSG00000000419        467        515        621        365        587
ENSG00000000457        260        211        263        164        245
ENSG00000000460         60         55         40         35         78
ENSG00000000938          0          0          2          0          1
                SRR1039517 SRR1039520 SRR1039521
ENSG00000000003       1047        770        572
ENSG00000000005          0          0          0
ENSG00000000419        799        417        508
ENSG00000000457        331        233        229
ENSG00000000460         63         76         60
ENSG00000000938          0          0          0
```

```
rowRanges(dds_raw)
```

```
GRangesList object of length 63677:
$ENSG00000000003
GRanges object with 17 ranges and 2 metadata columns:
       seqnames            ranges strand |   exon_id       exon_name
          <Rle>         <IRanges>  <Rle> | <integer>     <character>
   [1]        X 99883667-99884983      - |    667145 ENSE00001459322
   [2]        X 99885756-99885863      - |    667146 ENSE00000868868
```

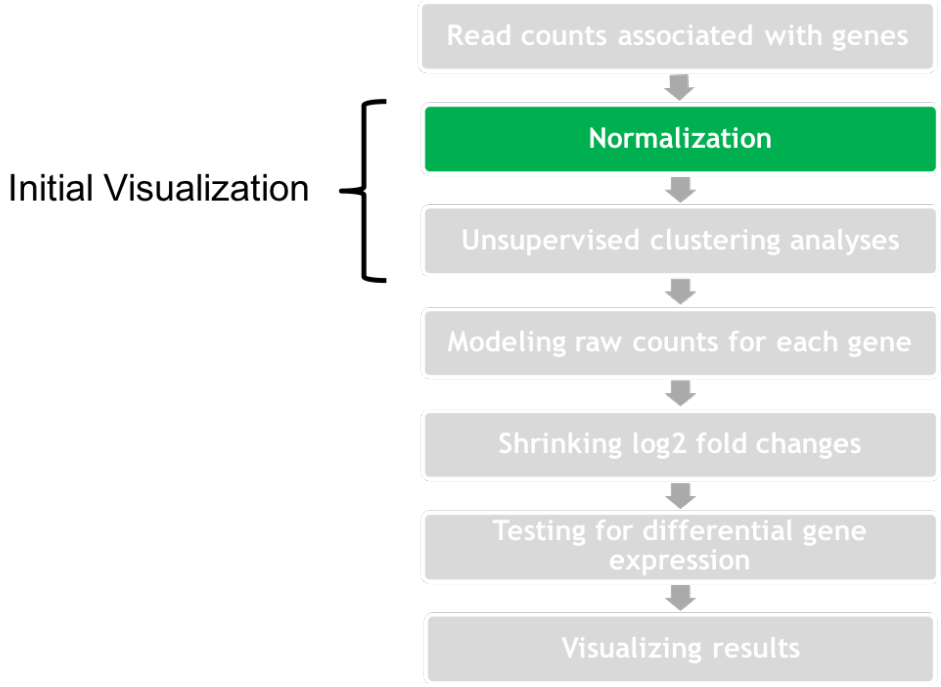
```
  [3]        X 99887482-99887565      - |    667147 ENSE00000401072
  [4]        X 99887538-99887565      - |    667148 ENSE00001849132
  [5]        X 99888402-99888536      - |    667149 ENSE00003554016
  ...      ...                 ...    ... .       ...             ...
 [13]        X 99890555-99890743      - |    667156 ENSE00003512331
 [14]        X 99891188-99891686      - |    667158 ENSE00001886883
 [15]        X 99891605-99891803      - |    667159 ENSE00001855382
 [16]        X 99891790-99892101      - |    667160 ENSE00001863395
 [17]        X 99894942-99894988      - |    667161 ENSE00001828996
 -------
 seqinfo: 722 sequences (1 circular) from an unspecified genome

...
<63676 more elements>
```

```
# Check object attributes and class
#attributes(dds)
class(dds_raw)
```

```
[1] "DESeqDataSet"
attr(,"package")
[1] "DESeq2"
```

1. Visual exploration of the data

There are two main paths on the RNA-seq differential expression analysis, first data exploration and second the proper DGE analysis. The **visualization** will imply to normalise the data in different ways and plot results to study the data distribution, correlation and clustering.



Gene count filtering

On our count table we have many genes with zero count. To reduce object size and computational time we can remove lowly expressed genes.

```
# Check counts on dds
head(counts(dds_raw))
```

```
                SRR1039508 SRR1039509 SRR1039512 SRR1039513 SRR1039516
ENSG00000000003        679        448        873        408       1138
ENSG00000000005          0          0          0          0          0
ENSG00000000419        467        515        621        365        587
ENSG00000000457        260        211        263        164        245
ENSG00000000460         60         55         40         35         78
ENSG00000000938          0          0          2          0          1
                SRR1039517 SRR1039520 SRR1039521
ENSG00000000003       1047        770        572
ENSG00000000005          0          0          0
ENSG00000000419        799        417        508
ENSG00000000457        331        233        229
ENSG00000000460         63         76         60
ENSG00000000938          0          0          0
```

```
# Genes removal with less than 10 reads on all the conditions. This will help DeSeq2 to perfo
# Evermore DeSeq2 DGE on lowly and highly express genes doesn't perform really good so it's b
# eliminate them.

dds_raw <- dds_raw[ rowSums(counts(dds_raw)) > 10, ]

# Check counts on dds after filtering
head(counts(dds_raw))
```

```
                SRR1039508 SRR1039509 SRR1039512 SRR1039513 SRR1039516
ENSG00000000003        679        448        873        408       1138
ENSG00000000419        467        515        621        365        587
ENSG00000000457        260        211        263        164        245
ENSG00000000460         60         55         40         35         78
ENSG00000000971       3251       3679       6177       4252       6721
ENSG00000001036       1433       1062       1733        881       1424
                SRR1039517 SRR1039520 SRR1039521
ENSG00000000003       1047        770        572
ENSG00000000419        799        417        508
ENSG00000000457        331        233        229
ENSG00000000460         63         76         60
ENSG00000000971      11027       5176       7995
ENSG00000001036       1439       1359       1109
```

Normalization

Sequencing depth normalization

```
# Investigate different sequencing library sizes. DGE software will use this sequencing
# depth information to normalise between samples.

colSums(counts(dds_raw))
```

```
SRR1039508 SRR1039509 SRR1039512 SRR1039513 SRR1039516 SRR1039517 SRR1039520
  20633673   18805036   25342811   15160577   24442836   30811815   19121708
SRR1039521
  21160391
```

```
# Calculate the size factor and add it to the data set.
dds_raw <- estimateSizeFactors(dds_raw)
sizeFactors(dds_raw)
```

```
SRR1039508 SRR1039509 SRR1039512 SRR1039513 SRR1039516 SRR1039517 SRR1039520
 1.0236767  0.8962366  1.1795988  0.6699588  1.1776734  1.3991183  0.9207797
SRR1039521
 0.9446040
```

```
# counts()  allows to immediately retrieve the **normalized** read counts.
counts_normalized  <- counts(dds_raw, normalized = TRUE)
# Now take a look at the sum of the total depth after normalization
colSums(counts(dds_raw, normalized=TRUE))
```

```
SRR1039508 SRR1039509 SRR1039512 SRR1039513 SRR1039516 SRR1039517 SRR1039520
  20156435   20982222   21484263   22629118   20755190   22022309   20766865
SRR1039521
  22401336
```

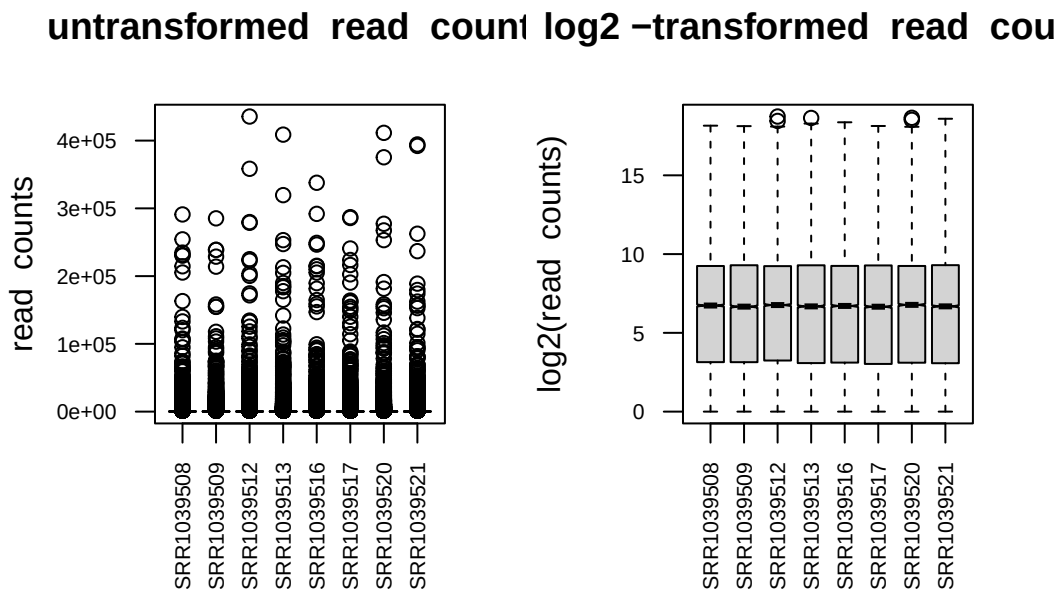
```
# Observe now the normalized counts
head(counts_normalized)
```

```
                SRR1039508 SRR1039509 SRR1039512 SRR1039513 SRR1039516
ENSG00000000003  663.29536  499.86798  740.08214  608.99265  966.31203
ENSG00000000419  456.19872  574.62502  526.45018  544.80960  498.44039
ENSG00000000457  253.98644  235.42889  222.95716  244.79116  208.03730
ENSG00000000460   58.61226   61.36772   33.90983   52.24202   66.23228
ENSG00000000971 3175.80737 4104.94265 5236.52621 6346.65873 5707.01505
ENSG00000001036 1399.85603 1184.95491 1469.14358 1315.00619 1209.16373
                SRR1039517 SRR1039520 SRR1039521
ENSG00000000003  748.32844  836.24781  605.54477
ENSG00000000419  571.07395  452.87706  537.79151
ENSG00000000457  236.57757  253.04641  242.42964
ENSG00000000460   45.02836   82.53874   63.51868
ENSG00000000971 7881.39228 5621.32292 8463.86436
ENSG00000001036 1028.50490 1475.92308 1174.03697
```

Log^2 transformation of read counts.

Log^2 transformation of the data make normalization easily interpretable and comparable. Simply using the log2 function, after adding 1 pseudocount, to avoid taking the log of zero.

```
# Log^2^ normalization adding 1 pseudocount.
counts_log_normalized <- log2(counts_normalized + 1)

par(mfrow=c(1,2)) # to plot the following two images side by side each other

# first , boxplots of non -transformed read counts (one per sample)
boxplot(counts_normalized , notch = TRUE , las=2, cex.axis = 0.7,
        main = "untransformed read counts", ylab = "read counts")

# box plots of log^2^ -transformed read counts
boxplot(counts_log_normalized , notch = TRUE , las=2, cex.axis = 0.7,
        main = "log2 -transformed read counts",
        ylab = "log2(read counts)")
```

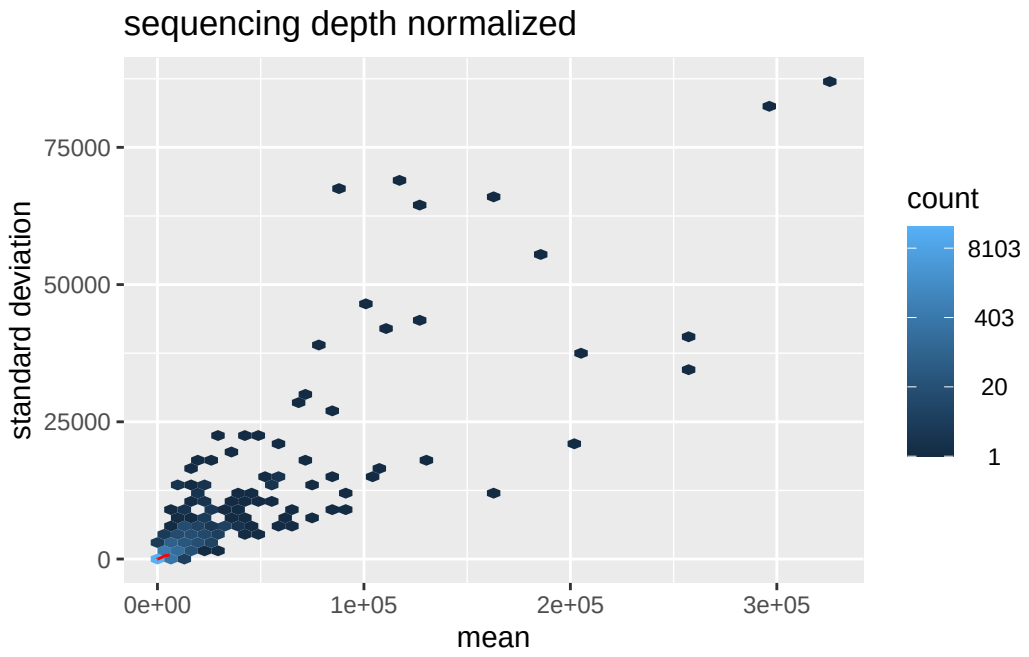


Data transformation

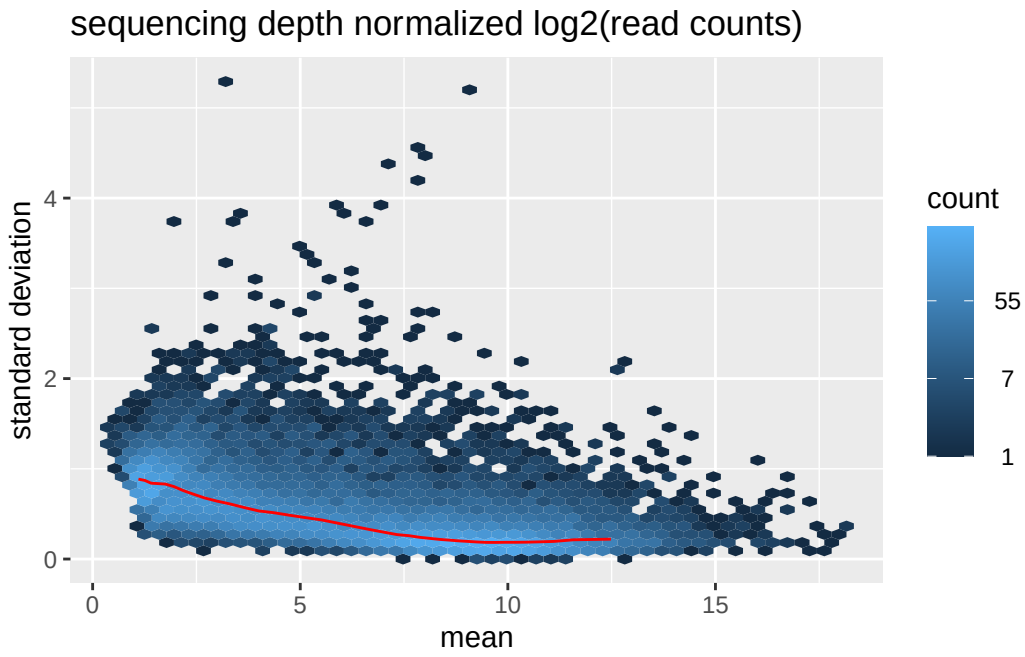
When the expected amount of variance is approximately the same across different mean values, the data is said to be homoskedastic. For RNA-seq counts, however, the expected variance grows with the mean.

```
# If needed, install the vsn package
# BiocManager::install("vsn")
```

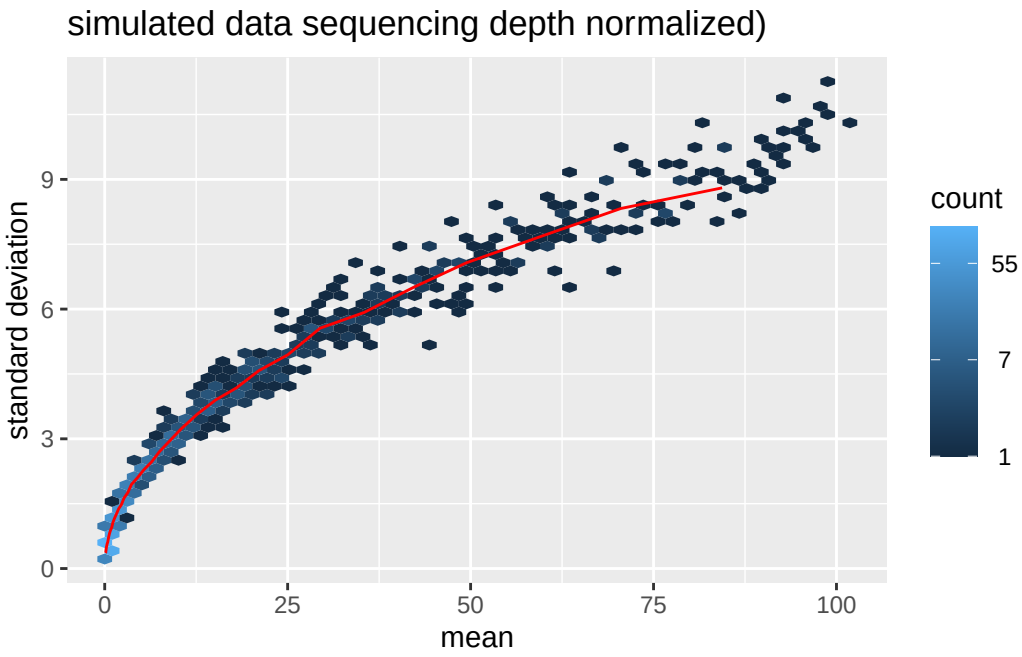
```r
#biocLite("vsn")
# if (!require(vsn)) {
#     install.packages("vsn")
#     require(vsn)
# }
library("vsn")

# If needed, install  the ggplot2 package
# BiocManager::install("ggplot2", force = TRUE)
# if (!require(ggplot2)) {
#     install.packages("ggplot2")
#     require(ggplot2)
# }
library(ggplot2)

# BiocManager::install("hexbin")
library("hexbin")


# When the expected amount of variance is approximately the same across different mean
# values, the data is said to be homoskedastic. For RNA-seq counts, however, the expected
# variance grows with the mean.

SdPlot <- meanSdPlot(counts_normalized, ranks = FALSE, plot = FALSE)

SdPlot$gg + ggtitle("sequencing depth normalized") + ylab("standard deviation")
```



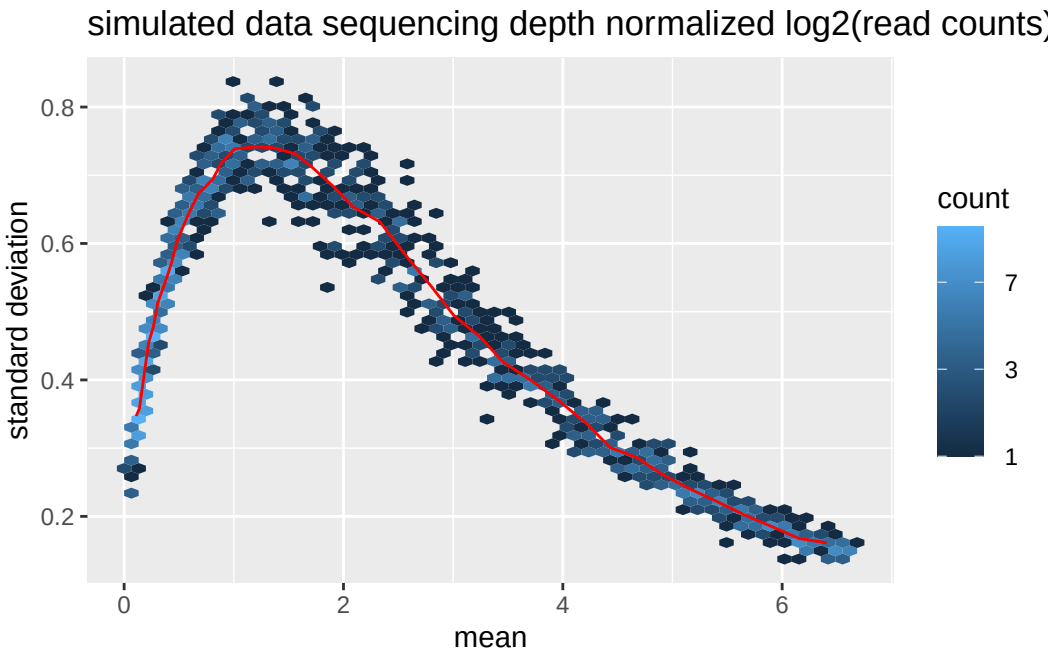

```
# The logarithm with a small pseudocount amplifies differences when the values are close
# to 0. The low count genes with low signal-to-noise ratio will overly contribute to
# sample-sample distances and PCA plots.

SdPlot_log <- meanSdPlot(counts_log_normalized, ranks = FALSE, plot = FALSE)
SdPlot_log$gg + ggtitle("sequencing depth normalized log2(read counts)") + ylab("standard dev
```




```
# We can see this property of count with simulated data. Poison counts in a range of lambda (
lambda <- 10^seq(from = -1, to = 2, length = 1000)
cts <- matrix(rpois(1000*100, lambda), ncol = 100)


# Standard deviation of each row against the mean on raw counts
sim_SdPlot <- meanSdPlot(cts, ranks = FALSE, plot = FALSE)
sim_SdPlot$gg + ggtitle("simulated data sequencing depth normalized)") + ylab("standard devia
```




```
# Standard deviation of each row against the mean on log2 pseudocount transformed data
log.cts.one <- log2(cts + 1)
sim_SdPlot_log <- meanSdPlot(log.cts.one, ranks = FALSE, plot = FALSE)
sim_SdPlot_log$gg + ggtitle("simulated data sequencing depth normalized log2(read counts)") +
```



DeSeq2 data transformation

As a solution, DESeq2 offers two transformations for count data that stabilize the variance across the mean: the variance stabilizing transformation (VST) for negative binomial data with a dispersion-mean trend (Anders and Huber 2010), implemented in the vst function, and the regularized-logarithm transformation or rlog (Love, Huber, and Anders 2014).

For genes with high counts, both the VST and the rlog will give similar result to the ordinary log2 transformation of normalized counts. For genes with lower counts, however, the values are shrunken towards a middle value. The VST or rlog-transformed data then become approximately homoskedastic (more flat trend in the meanSdPlot), and can be used directly for computing distances between samples, making PCA plots, or as input to downstream methods which perform best with homoskedastic data.

Which transformation to choose? The VST is much faster to compute and is less sensitive to high count outliers than the rlog. The rlog tends to work well on small datasets ($n < 30$), potentially outperforming the VST when there is a wide range of sequencing depth across samples (an order of magnitude difference). We therefore recommend the VST for medium-to-large datasets ($n > 30$). You can perform both transformations and compare the meanSdPlot or PCA plots generated, as described below.

Note that the two transformations offered by DESeq2 are provided for applications other than differential testing. For differential testing we recommend the DESeq function applied to **raw counts**, as described later in this workflow, which also takes into account

the dependence of the variance of counts on the mean value during the dispersion estimation step.

- **Variance stabilizing transformation**

```
vsd <- vst(dds_raw, blind = FALSE)
head(assay(vsd), 3)
```

```
                SRR1039508 SRR1039509 SRR1039512 SRR1039513 SRR1039516
ENSG00000000003   9.819191   9.523047   9.939055   9.727694  10.242102
ENSG00000000419   9.431670   9.666481   9.575795   9.611069   9.520155
ENSG00000000457   8.899577   8.837557   8.794049   8.869235   8.739868
                SRR1039517 SRR1039520 SRR1039521
ENSG00000000003   9.951334  10.075948   9.721674
ENSG00000000419   9.659998   9.424459   9.597692
ENSG00000000457   8.841488   8.896509   8.861321
```

- **The rlog**

```
rld <- rlog(dds_raw, blind = FALSE)
head(assay(rld), 3)
```

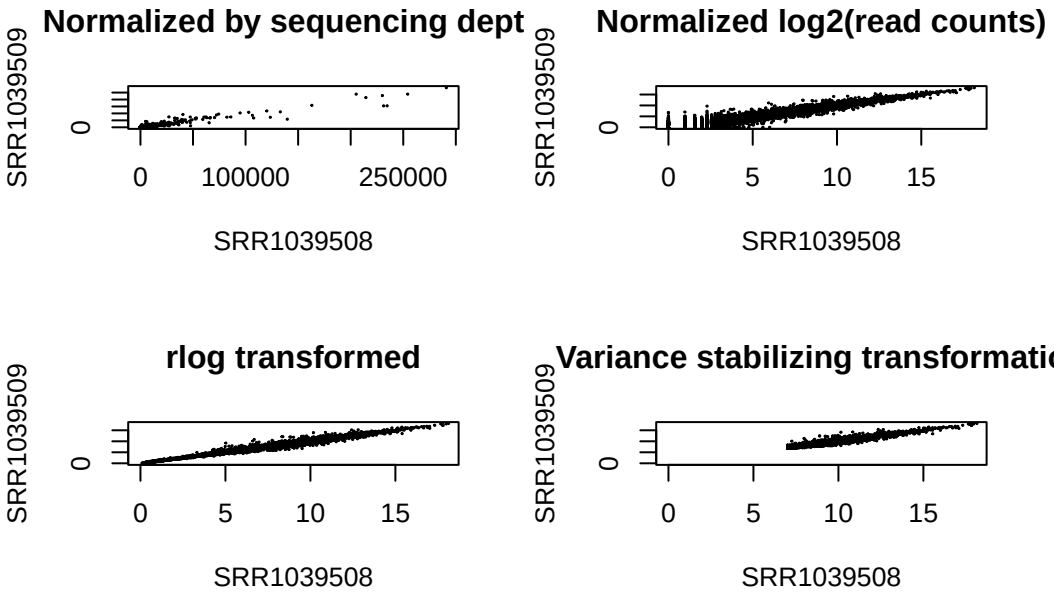
```
                SRR1039508 SRR1039509 SRR1039512 SRR1039513 SRR1039516
ENSG00000000003   9.385710   9.052987   9.516677   9.285691   9.838666
ENSG00000000419   8.869621   9.138138   9.035993   9.075431   8.972129
ENSG00000000457   7.961549   7.881275   7.823774   7.921721   7.751268
                SRR1039517 SRR1039520 SRR1039521
ENSG00000000003   9.530143   9.663011   9.277774
ENSG00000000419   9.131721   8.861576   9.060774
ENSG00000000457   7.886366   7.957315   7.912085
```

Sample correlation using all the different data transformations

We will study the correlation on the first two samples using different data transformations.

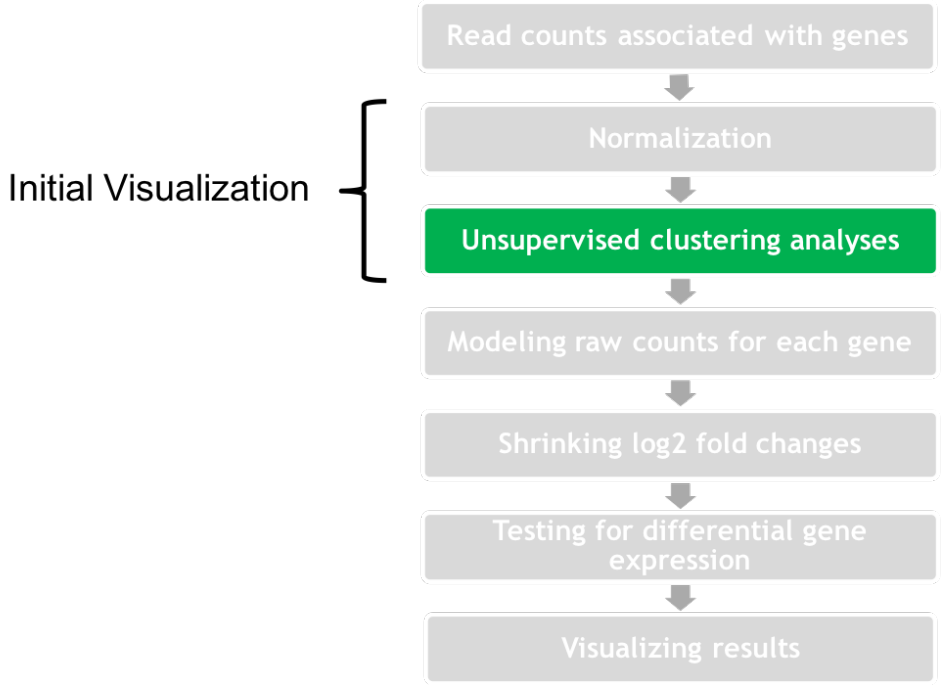
```
par(mfrow=c(2,2))
# Raw data normalized by sequencing depth
plot(counts_normalized [,1:2], cex=.1, main = "Normalized by sequencing depth")

# Log^2^ normalization adding 1 pseudocount.
plot(counts_log_normalized [,1:2], cex=.1, main = "Normalized log2(read counts)")
```

```
# rlog transformed
rlog_norm_counts <- assay(rld)
plot(rlog_norm_counts[,1:2], cex=.1, main = "rlog transformed", xlim=c(0,18), ylim=c(0,18))

# Variance stabilizing transformation
vsd_norm_counts <- assay(vsd)
plot(vsd_norm_counts[,1:2], cex=.1, main = "Variance stabilizing transformation", xlim=c(0,18
```



PCA & hierarchical clustering

Identifying outliers samples and sources of variation in the data.



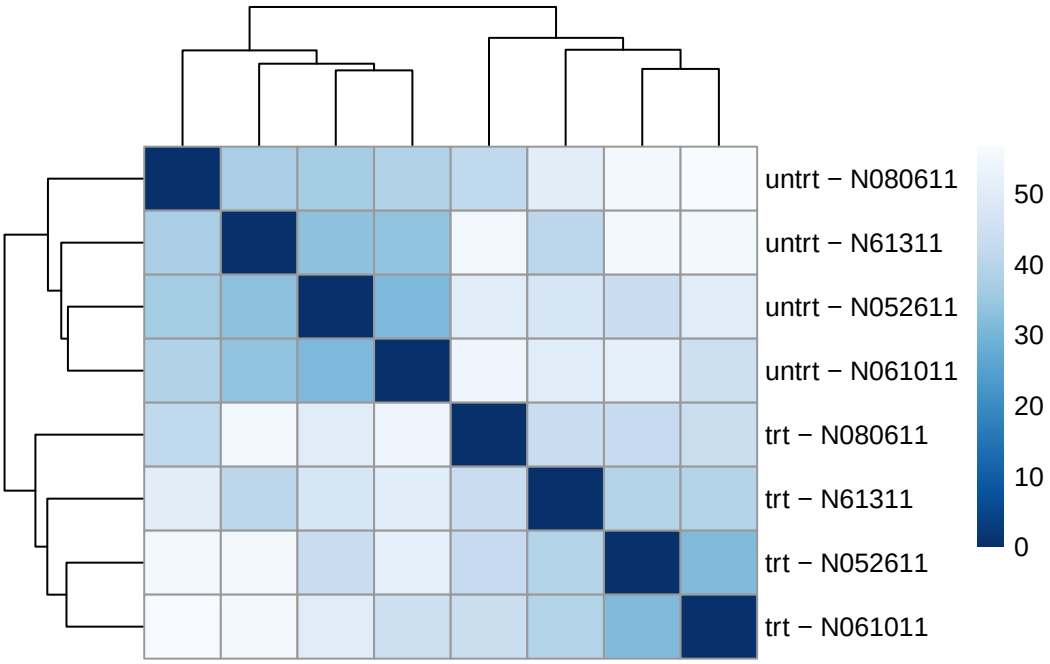
- **Variance stabilizing transformation**

```
# If needed, install the pheatmap and RColorBrewer packages
# BiocManager::install("pheatmap")
# if (!require(pheatmap)) {
#     install.packages("pheatmap")
#     require(pheatmap)
# }
library(pheatmap)

# BiocManager::install("RColorBrewer")
# if (!require(RColorBrewer)) {
#     install.packages("RColorBrewer")
#     require(RColorBrewer)
# }
library(RColorBrewer)

# Sample distances with VST
sampleDists_vsd <- dist(t(assay(vsd)))
sampleDists_vsd
```

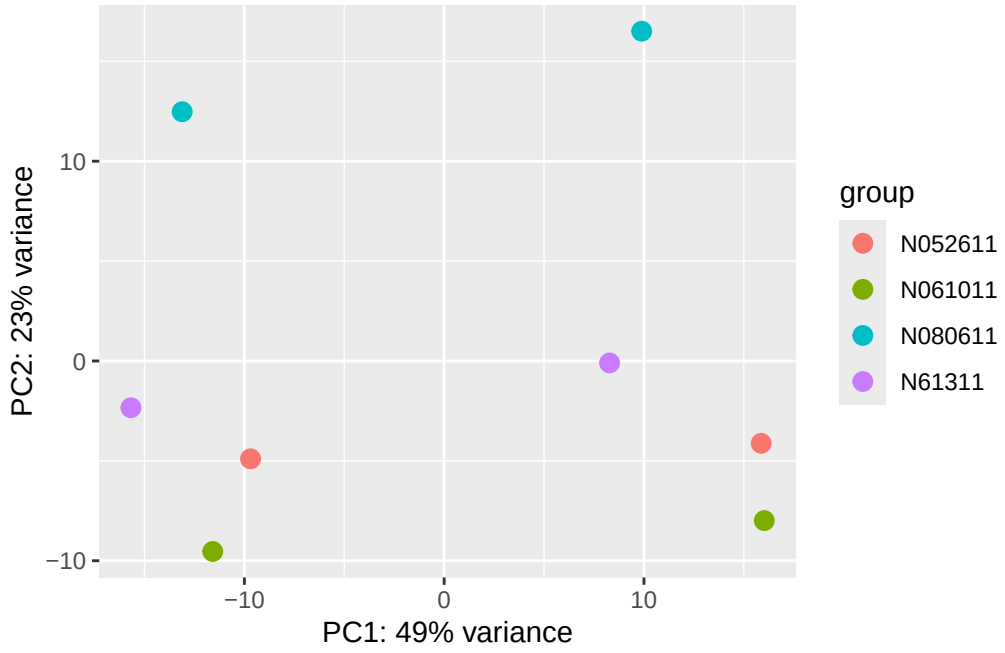
```
            SRR1039508 SRR1039509 SRR1039512 SRR1039513 SRR1039516 SRR1039517
```

```
SRR1039509   40.71891
SRR1039512   33.32015   47.68646
SRR1039513   55.32751   39.30305   43.74533
SRR1039516   37.41412   50.83267   36.64253   55.69234
SRR1039517   55.19399   43.71746   50.39249   42.60601   41.60202
SRR1039520   33.64095   50.03632   30.93601   51.49151   39.04477   54.24523
SRR1039521   55.43354   39.14690   50.37847   31.52966   56.64737   44.12058
           SRR1039520
SRR1039509
SRR1039512
SRR1039513
SRR1039516
SRR1039517
SRR1039520
SRR1039521   44.86001
```

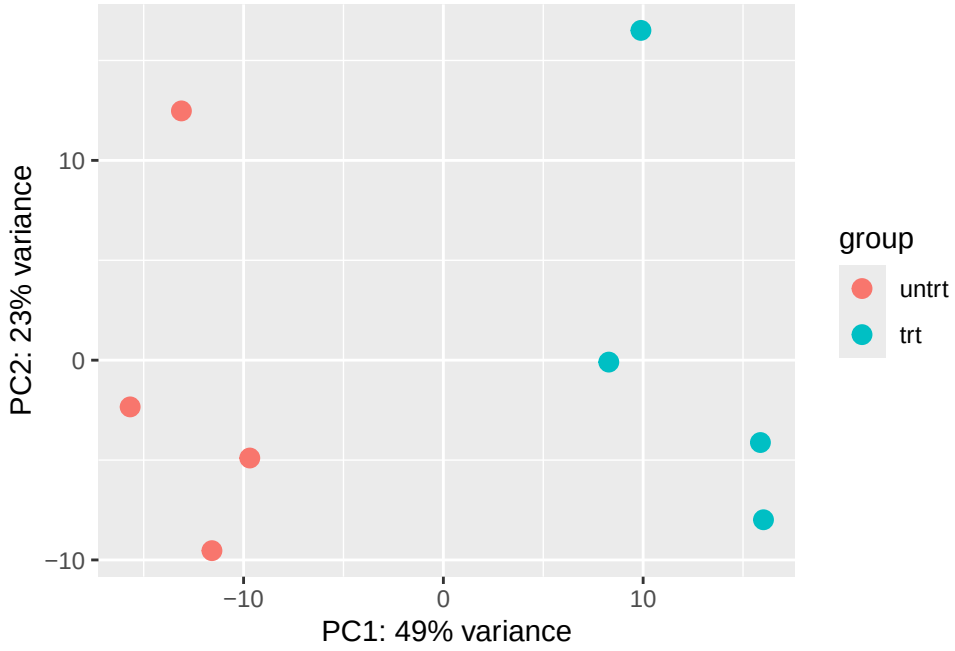
```
# Transform sample distances to matrix
sampleDistMatrix_vsd <- as.matrix( sampleDists_vsd )
# Modify row names joining treatment and cell type
rownames(sampleDistMatrix_vsd) <- paste( vsd$dex, vsd$cell, sep = " - " )
# Remove col names
colnames(sampleDistMatrix_vsd) <- NULL
# Colors palette
colors <- colorRampPalette( rev(brewer.pal(9, "Blues")) )(255)
# Draw heatmap
heatmap <- pheatmap(sampleDistMatrix_vsd,
             clustering_distance_rows = sampleDists_vsd,
             clustering_distance_cols = sampleDists_vsd,
             col = colors)
```



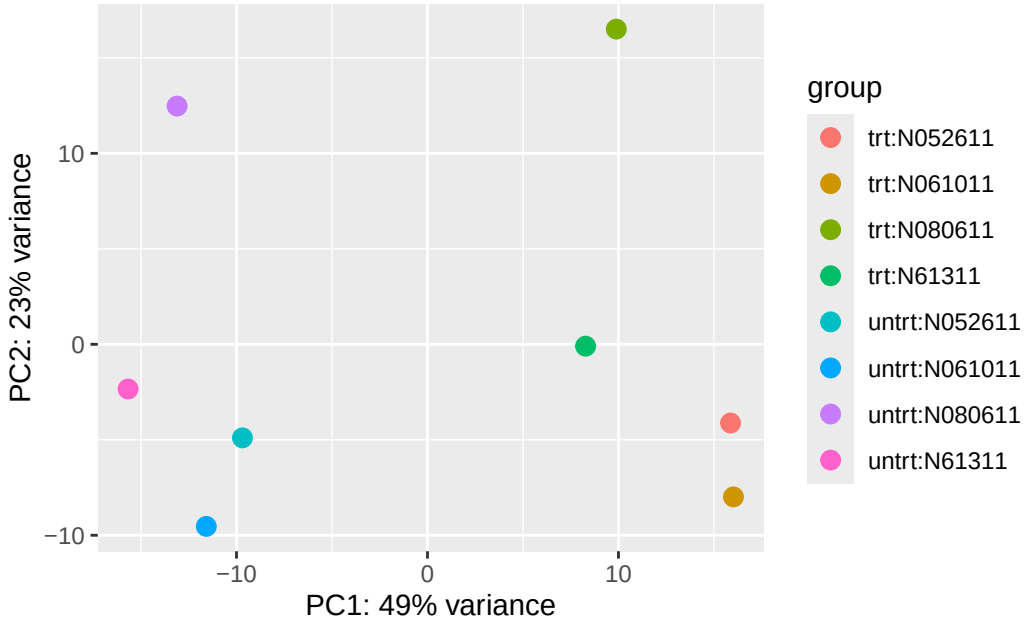

```
# Plot PCA on cell type
plotPCA(vsd, intgroup="cell")
```



```
# Plot PCA on dexamethasone treatment
plotPCA(vsd, intgroup="dex")
```




```
# Plot PCA on cell type and dexamethasone treatment
plotPCA(vsd, intgroup=c("dex", "cell"))
```

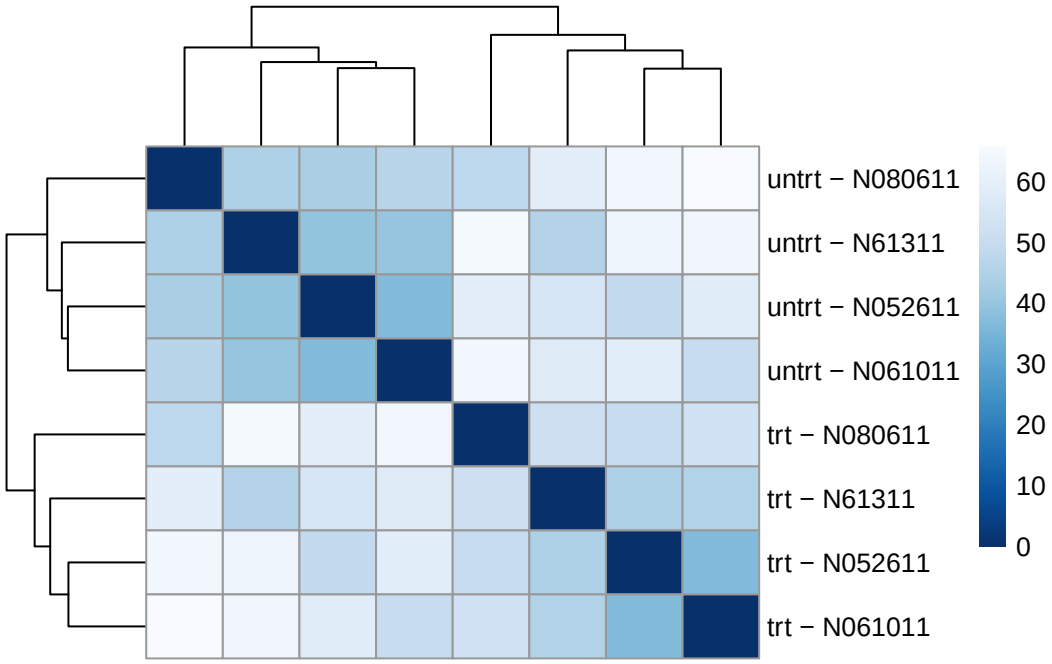


- **The rlog**

```
# Sample distances with rld
sampleDists_rld <- dist(t(assay(rld)))
sampleDists_rld
```

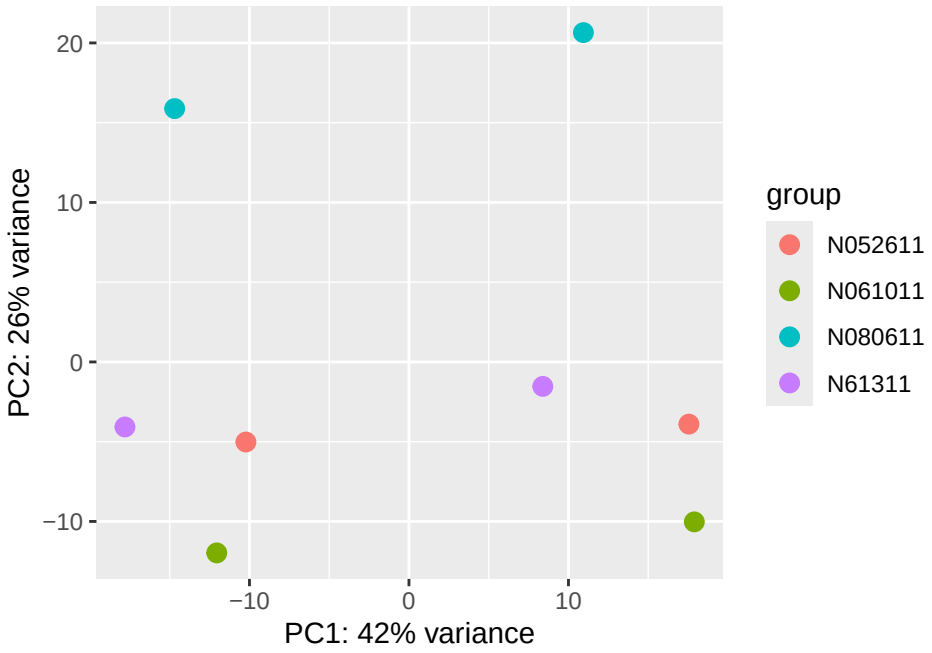
```
           SRR1039508 SRR1039509 SRR1039512 SRR1039513 SRR1039516 SRR1039517
SRR1039509   45.76449
SRR1039512   39.39689   55.05195
SRR1039513   62.75295   44.63162   48.80591
SRR1039516   44.67357   59.22327   43.73393   63.88698
SRR1039517   64.67402   51.61886   59.40815   50.04076   47.57564
SRR1039520   39.68018   57.57696   36.83867   58.59540   46.55154   63.76998
SRR1039521   63.48227   45.15104   57.99941   36.58571   65.69836   52.48221
           SRR1039520
SRR1039509
SRR1039512
SRR1039513
SRR1039516
SRR1039517
SRR1039520
SRR1039521   50.21932
```

```
# Transform sample distances to matrix
sampleDistMatrix_rld <- as.matrix( sampleDists_rld )
# Modify row names joining treatment and cell type
rownames(sampleDistMatrix_rld) <- paste( rld$dex, rld$cell, sep = " - " )
# Remove col names
colnames(sampleDistMatrix_rld) <- NULL
# Colors palette
colors <- colorRampPalette( rev(brewer.pal(9, "Blues")) )(255)
# Draw heatmap
heatmap <- pheatmap(sampleDistMatrix_rld,
              clustering_distance_rows = sampleDists_rld,
              clustering_distance_cols = sampleDists_rld,
              col = colors)
```



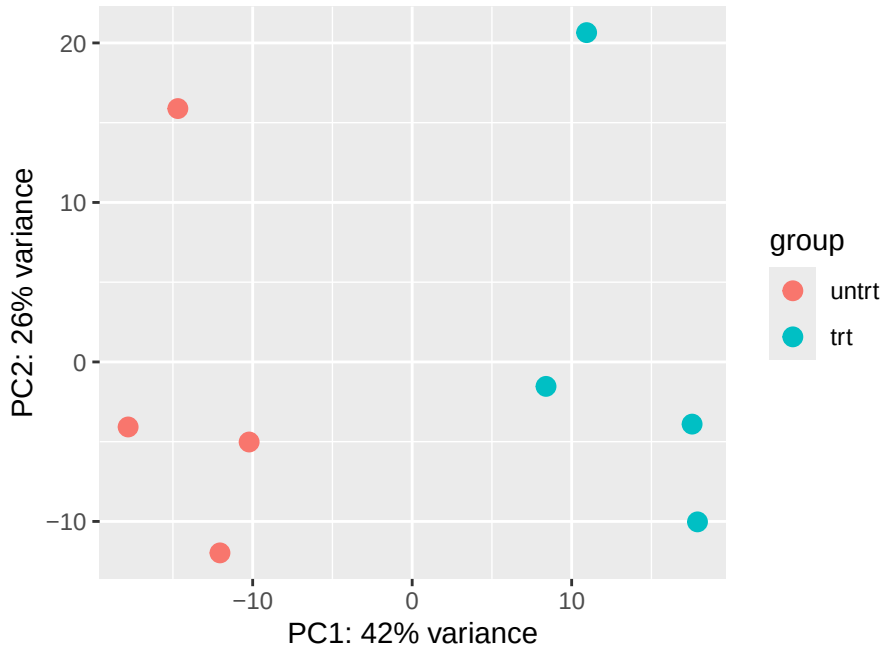

```
# Plot PCA on cell type
plotPCA(rld, intgroup="cell")
```

```
using ntop=500 top features by variance
```



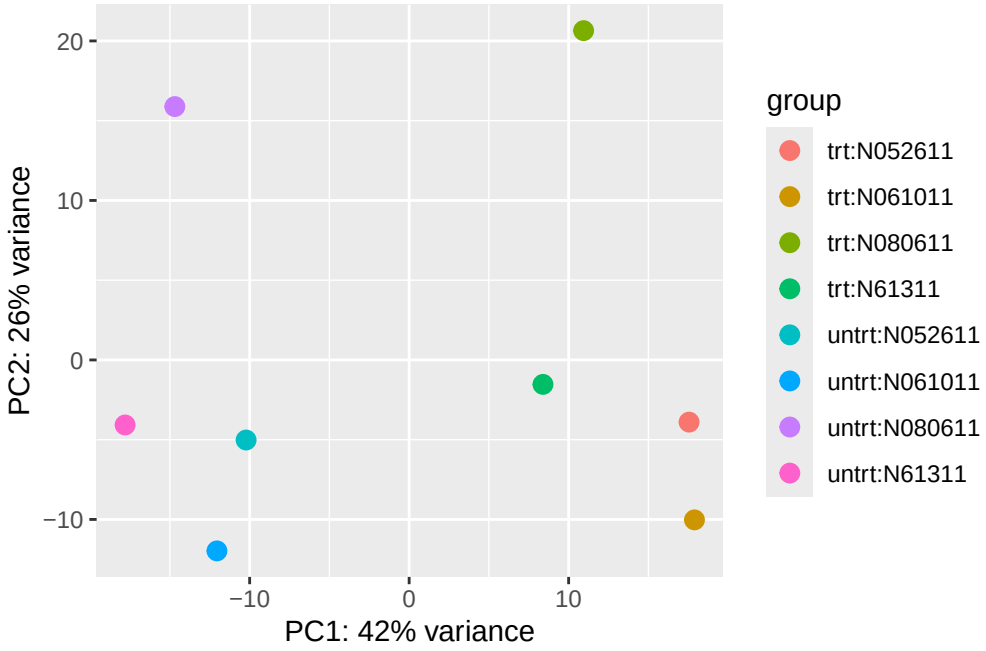

```
# Plot PCA on dexamethasone treatment
plotPCA(rld, intgroup="dex")
```

```
using ntop=500 top features by variance
```



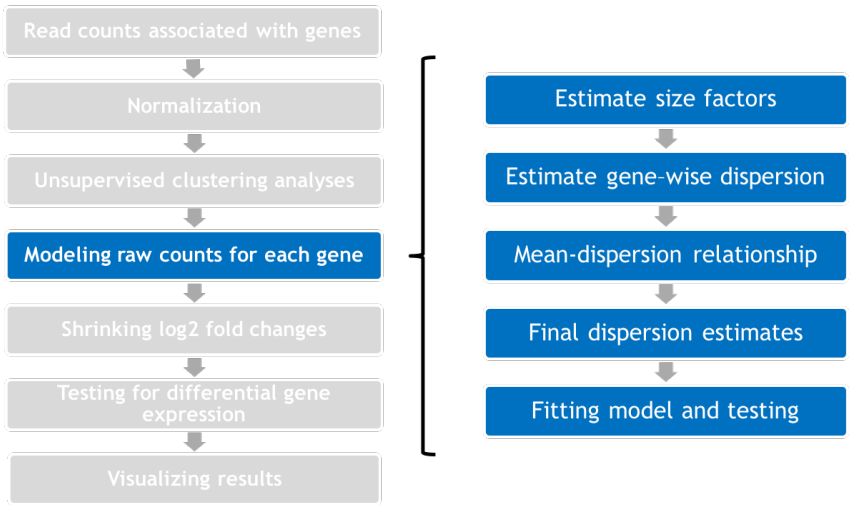

```
# Plot PCA on cell type and dexamethasone treatment
plotPCA(rld, intgroup=c("dex", "cell"))
```

```
using ntop=500 top features by variance
```



2. Differential gene expression

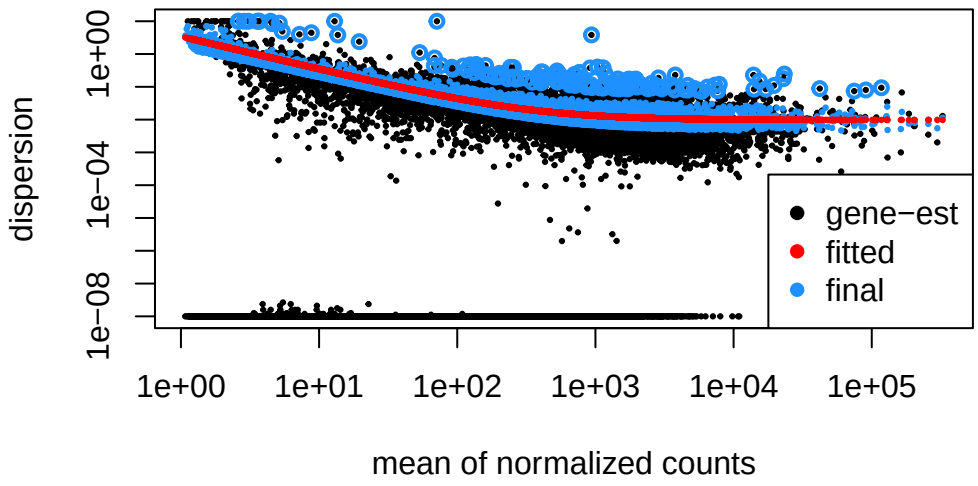
Now after the data exploration we can run the differential expression pipeline on the **raw counts** with a single call to the function DESeq. The null hypothesis is that there is no systematic difference between the average read count values of the different conditions for a given gene. We will calculate the fold change of read counts, assuming de differences in sequencing depth and variability. For out data we will like to test the effect of dexamethasone treatment versus the untreated cells (untreated used as denominator for the fold change calculation).




```
# Run DGE DeSeq2 pipeline
dds_DGE <- DESeq(dds_raw)
```

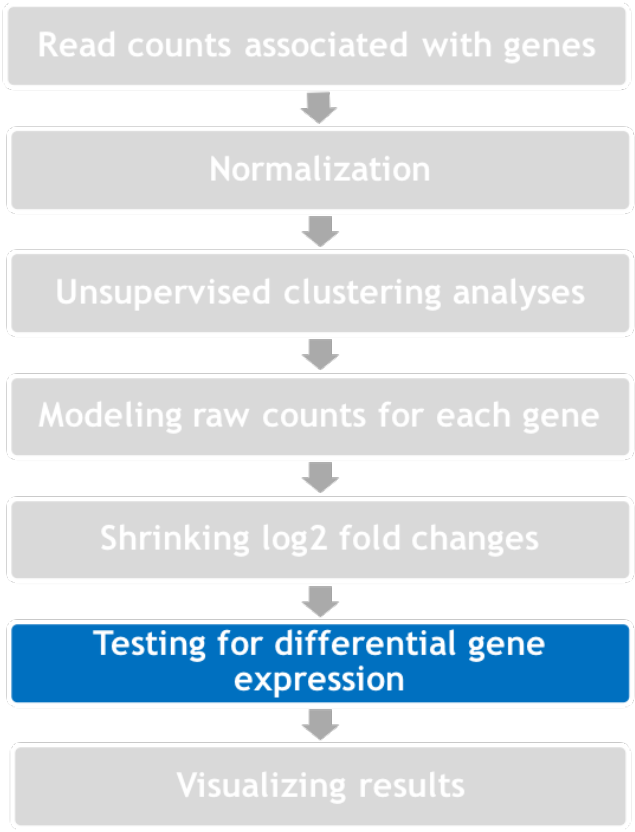
```
using pre-existing size factors

estimating dispersions

gene-wise dispersion estimates

mean-dispersion relationship

final dispersion estimates

fitting model and testing
```

```
# Mean-dispersion relationship
par(mfrow=c(1,1))
plotDispEsts(dds_DGE)
```



Building the results table

Now we can extract the results for the selected contrast, just calling results().




```
# Calling results() will build the base means across samples, log2 fold variation, standard e
# p value and p adjusted.

dds_DGE_results <- results(dds_DGE)
head(dds_DGE_results)
```

```
log2 fold change (MLE): dex trt vs untrt
Wald test p-value: dex trt vs untrt
DataFrame with 6 rows and 6 columns
                 baseMean log2FoldChange     lfcSE      stat      pvalue
                <numeric>      <numeric> <numeric> <numeric>   <numeric>
ENSG00000000003  708.5839     -0.3812438 0.1006569 -3.787556 1.52136e-04
ENSG00000000419  520.2833      0.2068250 0.1119673  1.847190 6.47195e-02
ENSG00000000457  237.1568      0.0379405 0.1425055  0.266239 7.90056e-01
ENSG00000000460   57.9312     -0.0887308 0.2839549 -0.312482 7.54674e-01
ENSG00000000971 5817.1912      0.4264088 0.0889024  4.796371 1.61566e-06
ENSG00000001036 1282.0737     -0.2410581 0.0890373 -2.707383 6.78160e-03
                     padj
```

```
                  <numeric>
ENSG00000000003 1.25654e-03
ENSG00000000419 1.91389e-01
ENSG00000000457 9.07267e-01
ENSG00000000460 8.89731e-01
ENSG00000000971 2.07705e-05
ENSG00000001036 3.30256e-02
```

```
# Result object can be filter like a data frame. We have 4023 significant genes with a p adju
table(dds_DGE_results$padj < 0.05)
```

```
FALSE  TRUE
13718  4023
```

```
# rownames(subset(dds_DGE_results, padj < 0.01))

# We can also access to the metadata contained on the columns and check the pairwise contrast
mcols(dds_DGE_results, use.names = TRUE)
```

```
DataFrame with 6 rows and 2 columns
                       type             description
                <character>             <character>
baseMean       intermediate mean of normalized c..
log2FoldChange      results log2 fold change (ML..
lfcSE               results standard error: dex ..
stat                results Wald statistic: dex ..
pvalue              results Wald test p-value: d..
padj                results   BH adjusted p-values
```

```
# Summary of DGE
summary(dds_DGE_results)
```

```
out of 22008 with nonzero total read count
adjusted p-value < 0.1
LFC > 0 (up)       : 2624, 12%
LFC < 0 (down)     : 2233, 10%
outliers [1]       : 0, 0%
low counts [2]     : 4267, 19%
```

```
(mean count < 5)
[1] see 'cooksCutoff' argument of ?results
[2] see 'independentFiltering' argument of ?results
```

```
# Select significant genes with a p adjusted lower than 0.05
resSig <- subset(dds_DGE_results, padj < 0.05)
# Order significant genes by p adjusted
resSig <- resSig[ order(resSig$padj), ]
# Order significant genes with the strongest down-regulation
head(resSig[ order(resSig$log2FoldChange), ])
```

```
log2 fold change (MLE): dex trt vs untrt
Wald test p-value: dex trt vs untrt
DataFrame with 6 rows and 6 columns
                 baseMean log2FoldChange     lfcSE      stat      pvalue
                <numeric>      <numeric> <numeric> <numeric>   <numeric>
ENSG00000128285   6.62454       -5.32504  1.248932  -4.26368 2.01091e-05
ENSG00000267339  26.23285       -4.61120  0.668404  -6.89882 5.24356e-12
ENSG00000019186  14.08722       -4.32425  0.850515  -5.08427 3.69048e-07
ENSG00000183454   5.80405       -4.26288  1.157619  -3.68246 2.30997e-04
ENSG00000146006  46.80589       -4.20876  0.525353  -8.01131 1.13496e-15
ENSG00000141469  53.43499       -4.12475  1.129823  -3.65079 2.61436e-04
                      padj
                 <numeric>
ENSG00000128285 2.06575e-04
ENSG00000267339 1.47895e-10
ENSG00000019186 5.36035e-06
ENSG00000183454 1.82058e-03
ENSG00000146006 4.89910e-14
ENSG00000141469 2.03427e-03
```

```
# And with the strongest up-regulation
head(resSig[ order(resSig$log2FoldChange, decreasing = TRUE), ])
```

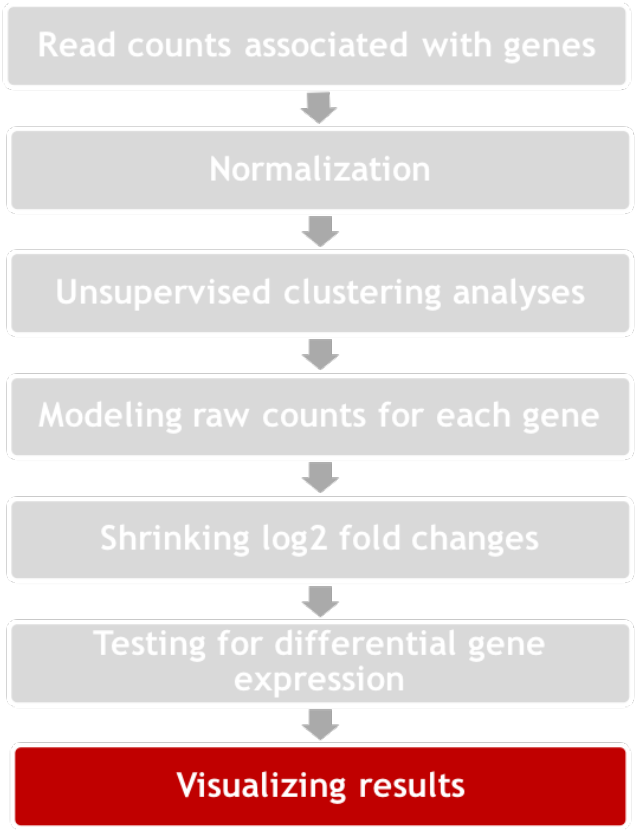
```
log2 fold change (MLE): dex trt vs untrt
Wald test p-value: dex trt vs untrt
DataFrame with 6 rows and 6 columns
                 baseMean log2FoldChange     lfcSE      stat      pvalue
                <numeric>      <numeric> <numeric> <numeric>   <numeric>
ENSG00000179593  67.24111        9.50598  1.053319   9.02479 1.80039e-19
ENSG00000109906 385.05722        7.35264  0.536394  13.70755 9.14995e-43
```

```
ENSG00000250978  56.31694        6.32629  0.675580   9.36424 7.66013e-21
ENSG00000132518   5.65448        5.88491  1.312168   4.48488 7.29565e-06
ENSG00000127954 286.39779        5.20720  0.493144  10.55919 4.60624e-26
ENSG00000249364   8.83902        5.09043  1.150576   4.42424 9.67817e-06
                       padj
                  <numeric>
ENSG00000179593 1.12864e-17
ENSG00000109906 2.28633e-40
ENSG00000250978 5.45777e-19
ENSG00000132518 8.26515e-05
ENSG00000127954 5.04440e-24
ENSG00000249364 1.06978e-04
```

```
# Save significant DGE results to file
write.table(resSig, file = "DESeq2_Sig_results_dex_Vs_untrt.tab", sep = "\t", quote = FALSE
```

Exploratory plots

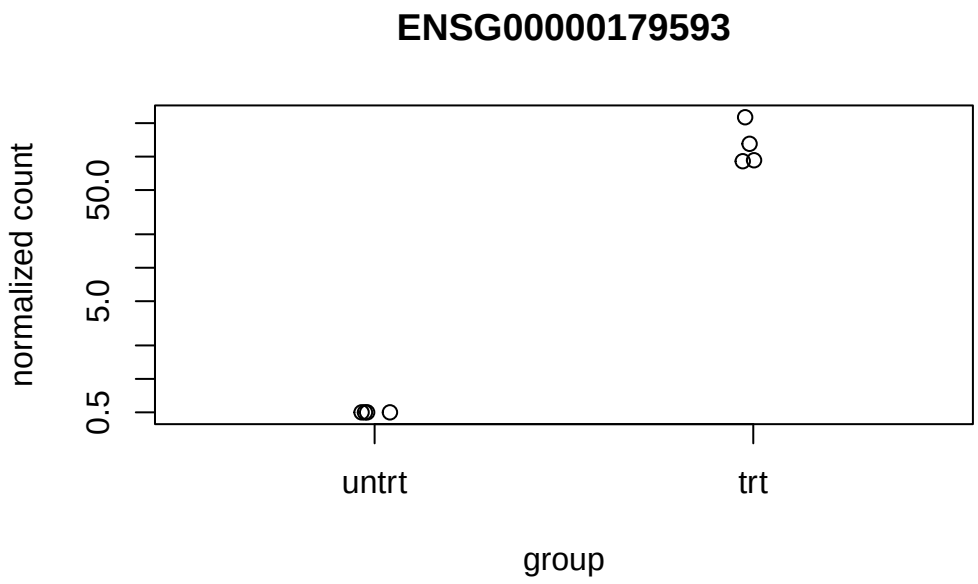
Plots are the best tools to explain DGE and your report should include them selecting those that best describe your findings.



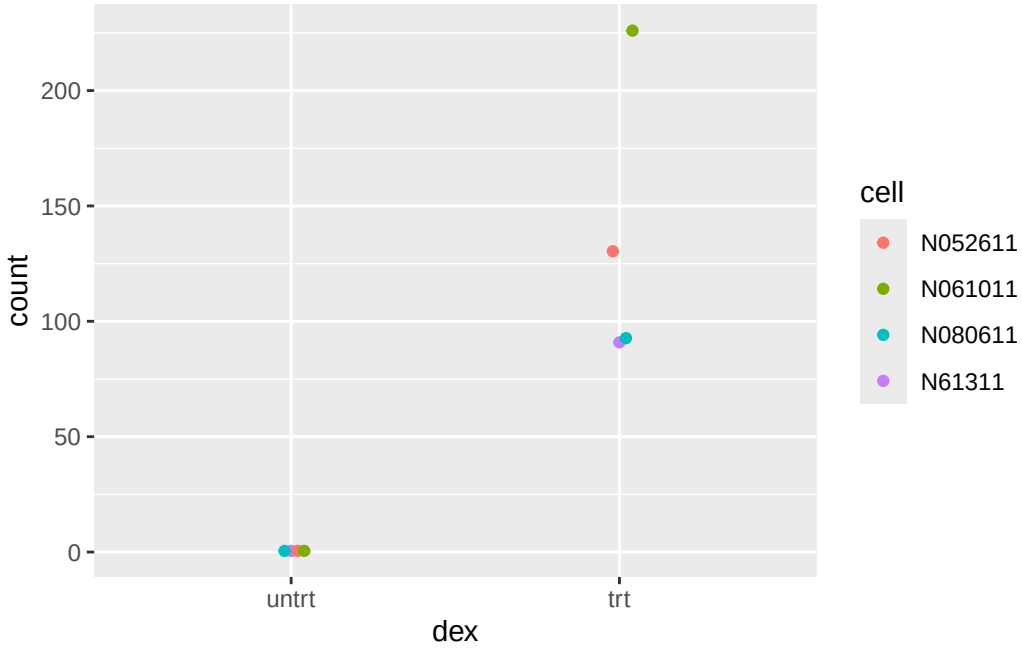
Individual gene count plot

A quick way to visualize the counts for a particular gene is to use the plotCounts function that takes as arguments the DESeqDataSet, a gene name, and the group over which to plot the counts (figure below).

```
# Individual gene raw count plot
plotCounts(dds_DGE, gene = "ENSG00000179593", intgroup=c("dex"))
```

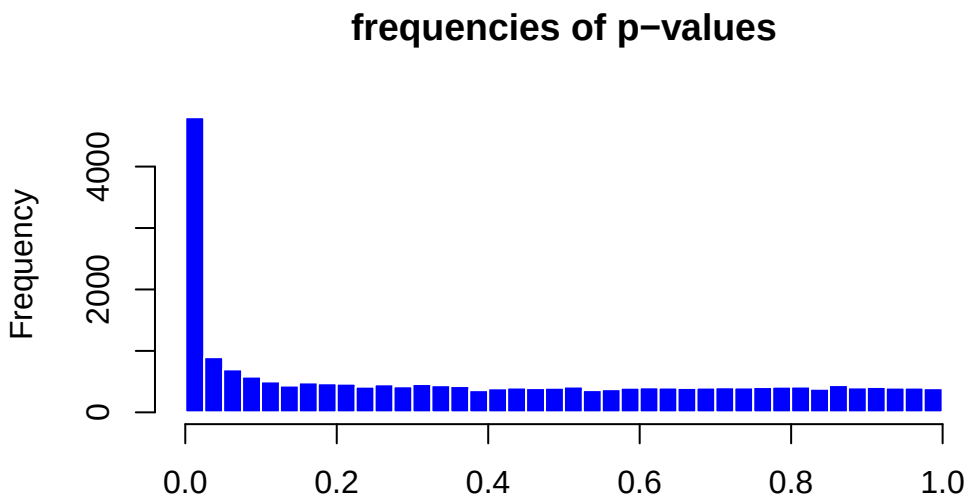



```
# Normalized counts for a single gene over treatment group.
# We can also make custom plots using the ggplot function from the ggplot2 package (figures l

# BiocManager::install("ggbeeswarm")
library("ggbeeswarm")
geneCounts <- plotCounts(dds_DGE, gene = "ENSG00000179593", intgroup = c("dex","cell"),
                         returnData = TRUE)
ggplot(geneCounts, aes(x = dex, y = count, color = cell)) +
    geom_beeswarm(cex = 1)
```



Histogram of frequencies

```
par(mfrow=c(1,1))
# Histogram of p-values frequencies
hist(dds_DGE_results$pvalue , col = "blue",  xlab = "", , border = "white", ylab = "Frequency
```



MA plot

An MA-plot (Dudoit et al. 2002) provides a useful overview for the distribution of the estimated coefficients in the model, e.g. the comparisons of interest, across all genes. On the y-axis, the "M" stands for "minus" – subtraction of log values is equivalent to the log of the ratio – and on the x-axis, the "A" stands for "average". This plot allows us to evaluate fold changes and the distribution around the mean expression.

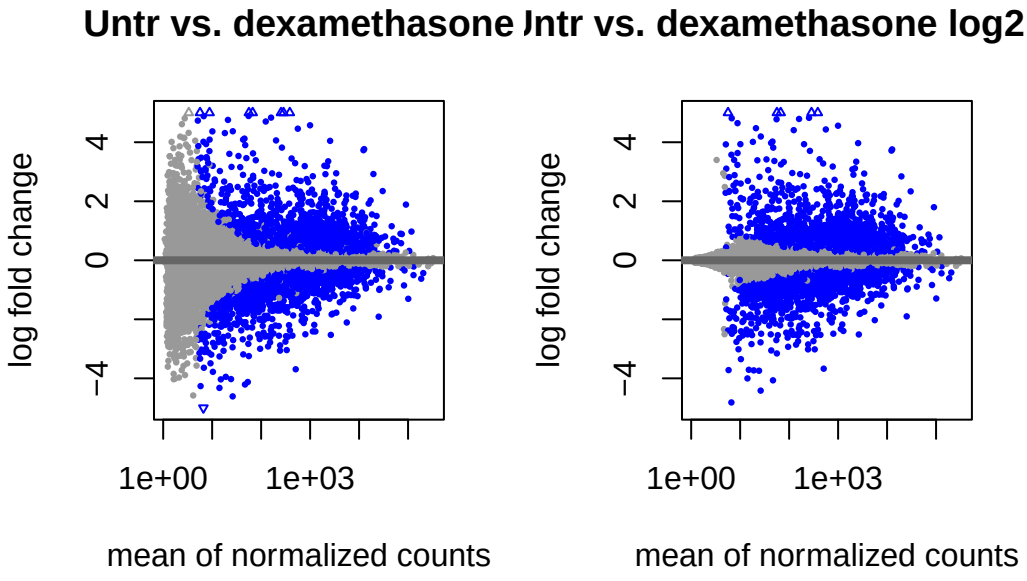
Before making the MA-plot, we use the lfcShrink function to shrink the log2 fold changes for the comparison of dex treated vs untreated samples. There are three types of shrinkage estimators in DESeq2, which are covered in the DESeq2 vignette. log2 fold shrinkage remove the noise preserving the large differences.

```
par(mfrow=c(1,2))
# MA plot without shrinkage
plotMA(dds_DGE_results, alpha = 0.05,  main = "Untr vs. dexamethasone", ylim = c(-5,5))

# Log^2^ fold shrinkage
# BiocManager::install("apeglm")
# if (!require(apeglm)) {
#     install.packages("apeglm")
#     require(apeglm)
# }
```

```
library("apeglm")
# Check possible contrast to create the MA plot
resultsNames(dds_DGE)
```

```
[1] "Intercept"               "cell_N061011_vs_N052611"
[3] "cell_N080611_vs_N052611" "cell_N61311_vs_N052611"
[5] "dex_trt_vs_untrt"
```

```
res <- lfcShrink(dds_DGE, coef="dex_trt_vs_untrt", type="apeglm")
plotMA(res, alpha = 0.05, main = "Untr vs. dexamethasone log2 shrink",ylim = c(-5, 5))
```

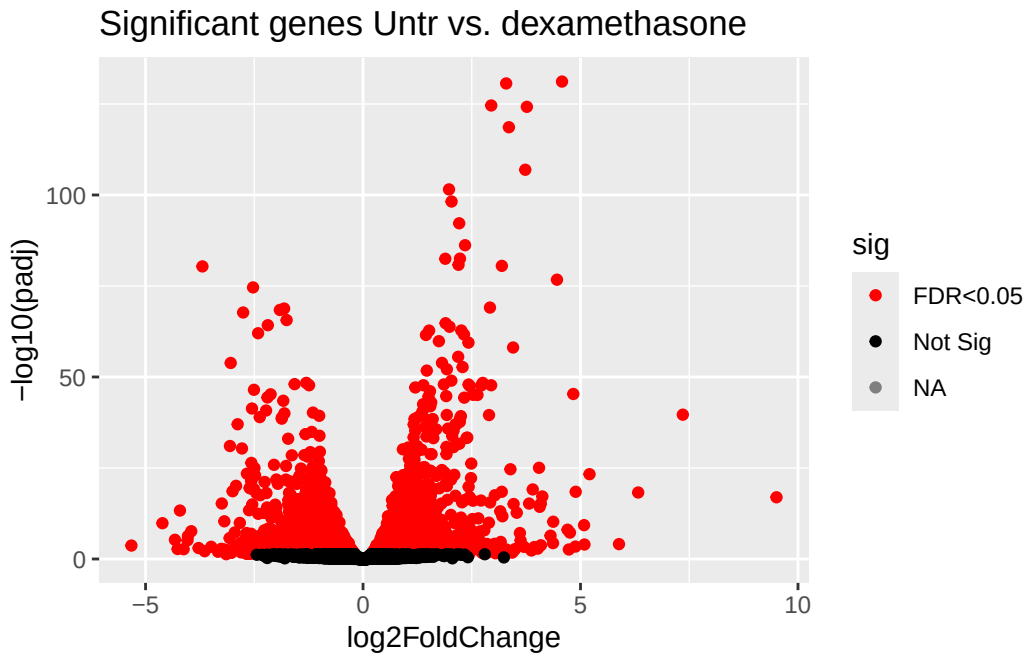


Volcano plot

Volcano plot helps to visualize significant differential expressed genes showing the $\log^2$ fold change and the significance (p-adjusted).

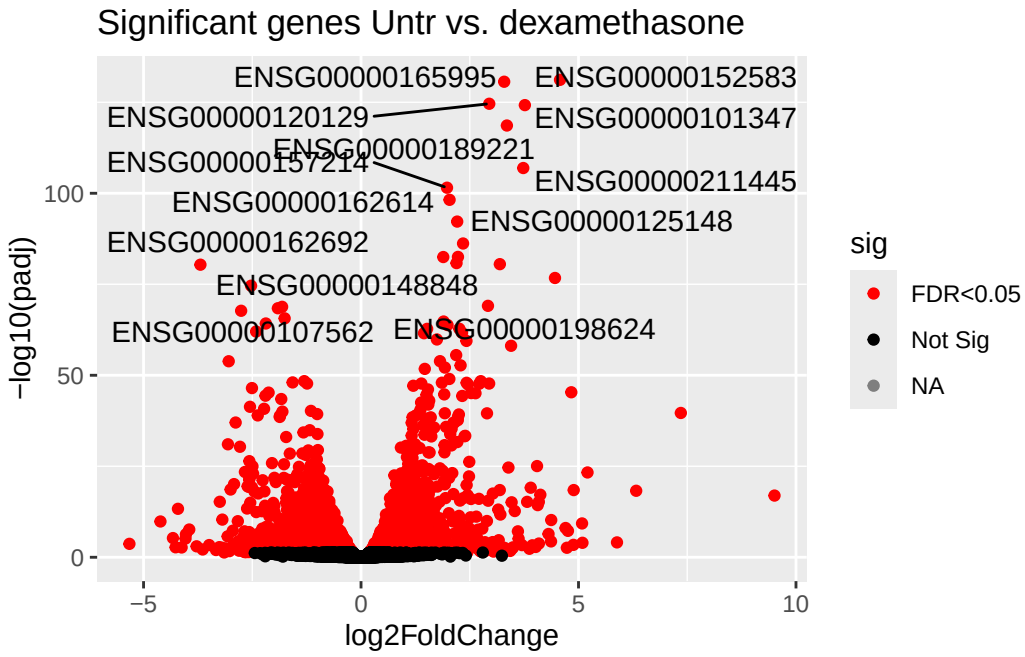
```
# BiocManager::install("dplyr")
# if (!require(dplyr)) {
#     install.packages("dplyr")
#     require(dplyr)
# }
```

```r
library("dplyr")

dds_DGE_results <- dds_DGE_results[order(dds_DGE_results$padj),]
results_order <- as.data.frame(dplyr::mutate(as.data.frame(dds_DGE_results), sig=ifelse(dds_

p = ggplot2::ggplot(results_order, ggplot2::aes(log2FoldChange, -log10(padj))) +
  ggplot2::geom_point(ggplot2::aes(col = sig)) +
  ggplot2::scale_color_manual(values = c("red", "black")) +
  ggplot2::ggtitle("Significant genes Untr vs. dexamethasone")

p
```



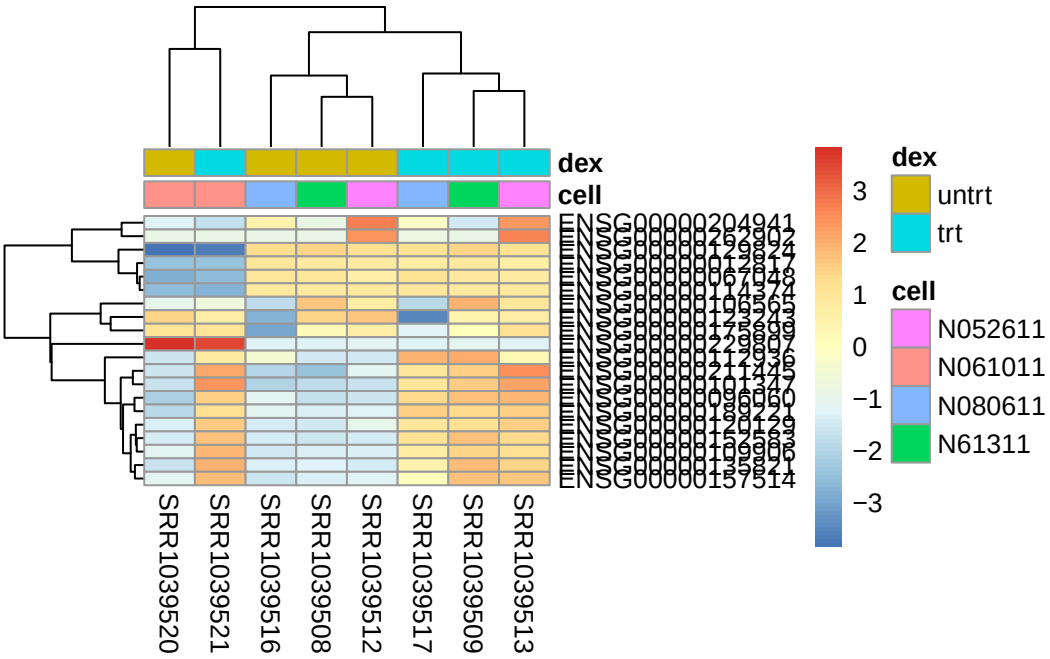

```r
# Get ENSEMBL gene names
# Grepel library helps to no overlap gene names
# BiocManager::install("ggrepel")
# if (!require(ggrepel)) {
#     install.packages("ggrepel")
#     require(ggrepel)
# }
library("ggrepel")
# Select genes that have log2 fold change higher than 2 and p adjusted lower than 0.05
```

```
DEgenes_DESeq <- results_order[which(abs(results_order$log2FoldChange) > log2(2) & results_or
DEgenes_DESeq <- DEgenes_DESeq[order(DEgenes_DESeq$padj),]

# Plot adding
p + ggrepel::geom_text_repel(data=DEgenes_DESeq[1:20, ], ggplot2::aes(label=rownames(results_
```



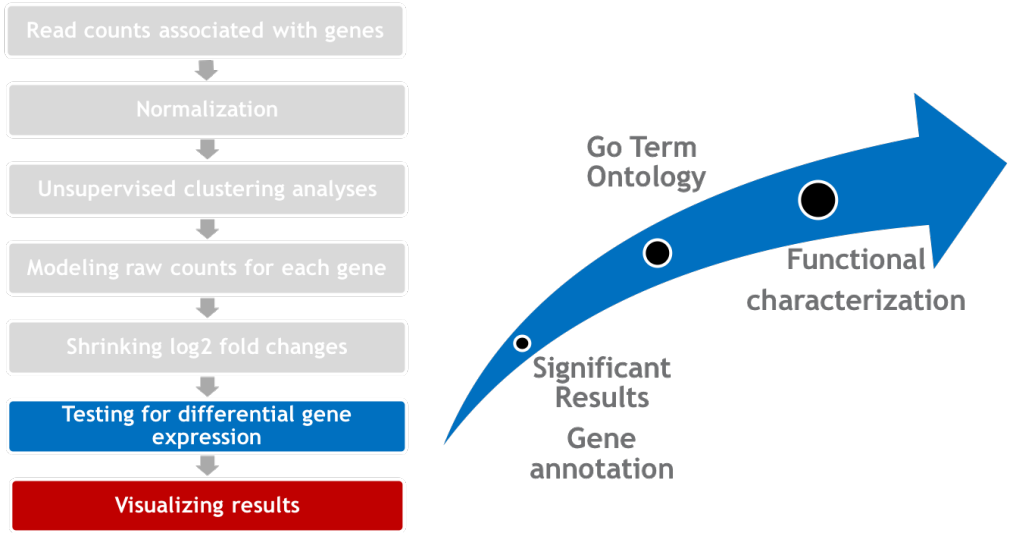
Heatmap plot

```
# Select the 20 most variable genes
topVarGenes <- head(order(rowVars(assay(vsd)), decreasing = TRUE), 20)

# Transform to matrix and use vsd transformation
mat  <- assay(vsd)[ topVarGenes, ]
mat  <- mat - rowMeans(mat)
anno <- as.data.frame(colData(vsd)[, c("cell","dex")])
pheatmap(mat, annotation_col = anno)
```



3. Annotate results

Our results data frame contains only ENSEMBL gene ID, but we need some more meaning full info. For that purpose we will add gene symbol, gene name, KEEG path and ENTREZ ID.



```
# Load AnnotationDbi package https://bioconductor.org/packages/3.9/AnnotationDbi
# BiocManager::install("AnnotationDbi")
# if (!require(AnnotationDbi)) {
#     install.packages("AnnotationDbi")
#     require(AnnotationDbi)
# }
library("AnnotationDbi")

# Load org.Hs.eg.db package https://bioconductor.org/packages/3.9/org.Hs.eg.db
# BiocManager::install("org.Hs.eg.db")
# if (!require(org.Hs.eg.db)) {
#     install.packages("org.Hs.eg.db")
#     require(org.Hs.eg.db)
# }
library("org.Hs.eg.db")

# Check all the possible terms that could be retrieved with AnnotationDbi "mapsIds function"
columns(org.Hs.eg.db)
```

```
 [1] "ACCNUM"       "ALIAS"        "ENSEMBL"      "ENSEMBLPROT"  "ENSEMBLTRANS"
 [6] "ENTREZID"     "ENZYME"       "EVIDENCE"     "EVIDENCEALL"  "GENENAME"
[11] "GENETYPE"     "GO"           "GOALL"        "IPI"          "MAP"
[16] "OMIM"         "ONTOLOGY"     "ONTOLOGYALL"  "PATH"         "PFAM"
[21] "PMID"         "PROSITE"      "REFSEQ"       "SYMBOL"       "UCSCKG"
[26] "UNIPROT"
```

```
# Ordered signifcant genes
head(resSig)
```

```
log2 fold change (MLE): dex trt vs untrt
Wald test p-value: dex trt vs untrt
DataFrame with 6 rows and 6 columns
                 baseMean log2FoldChange     lfcSE      stat       pvalue
                <numeric>      <numeric> <numeric> <numeric>    <numeric>
ENSG00000152583   997.410        4.57495  0.184224   24.8337 3.87953e-136
ENSG00000165995   495.089        3.29110  0.132933   24.7576 2.56857e-135
ENSG00000120129  3408.961        2.94784  0.121952   24.1721 4.37074e-129
ENSG00000101347 12703.198        3.76701  0.156145   24.1250 1.36567e-128
ENSG00000189221  2341.724        3.35365  0.142252   23.5754 6.89933e-123
ENSG00000211445 12285.646        3.73043  0.166538   22.3999 3.94527e-111
                      padj
```

```
                    <numeric>
ENSG00000152583 6.88268e-132
ENSG00000165995 2.27845e-131
ENSG00000120129 2.58471e-125
ENSG00000101347 6.05707e-125
ENSG00000189221 2.44802e-119
ENSG00000211445 1.16655e-107
```

```
# Add gene symbol column
resSig$SYMBOL = mapIds(org.Hs.eg.db,
                       keys=rownames(resSig),
                       column="SYMBOL",
                       keytype="ENSEMBL",
                       multiVals="first")

# Add gene name column
resSig$GENENAME = mapIds(org.Hs.eg.db,
                       keys=rownames(resSig),
                       column="GENENAME",
                       keytype="ENSEMBL",
                       multiVals="first")

# Add functional path column
resSig$PATH = mapIds(org.Hs.eg.db,
                       keys=rownames(resSig),
                       column="PATH",
                       keytype="ENSEMBL",
                       multiVals="first")

# Add entrex ID column
resSig$ENTREZID = mapIds(org.Hs.eg.db,
                       keys=rownames(resSig),
                       column="ENTREZID",
                       keytype="ENSEMBL",
                       multiVals="first")



# Add ENSEMBL column
resSig$ENSEMBL <- rownames(resSig)

resSig_DF <- as.data.frame(resSig)
```

```
# Save annotated data frame
write.table(resSig_DF, file=("Significant_DE_dex_trt_vs_untrt_metadata.tab"), row.names = F,
```

4. Functional Gene Ontology (GO) analysis

Harvard Chan Bioinformatics Core (HBC) - https://github.com/hbctraining/DGE_workshop/blob/master/lessons/09_functional_analysis.md
g:Profiler - http://biit.cs.ut.ee/gprofiler/index.cgi
DAVID - http://david.abcc.ncifcrf.gov/tools.jsp
clusterProfiler - http://bioconductor.org/packages/release/bioc/html/clusterProfiler.html

```
# Load clusterProfiler package  http://bioconductor.org/packages/release/bioc/html/clusterPro
# BiocManager::install("clusterProfiler")
# if (!require(clusterProfiler)) {
#     install.packages("clusterProfiler")
#     require(clusterProfiler)
# }
library("clusterProfiler")
```

```
clusterProfiler v4.18.4 Learn more at https://yulab-smu.top/contribution-
knowledge-mining/

Please cite:

S Xu, E Hu, Y Cai, Z Xie, X Luo, L Zhan, W Tang, Q Wang, B Liu, R Wang,
W Xie, T Wu, L Xie, G Yu. Using clusterProfiler to characterize
multiomics data. Nature Protocols. 2024, 19(11):3292-3320


Attaching package: 'clusterProfiler'

The following object is masked from 'package:AnnotationDbi':

    select

The following object is masked from 'package:IRanges':

    slice
```

```
The following object is masked from 'package:S4Vectors':

    rename

The following object is masked from 'package:stats':

    filter
```

```
library("enrichplot")
```

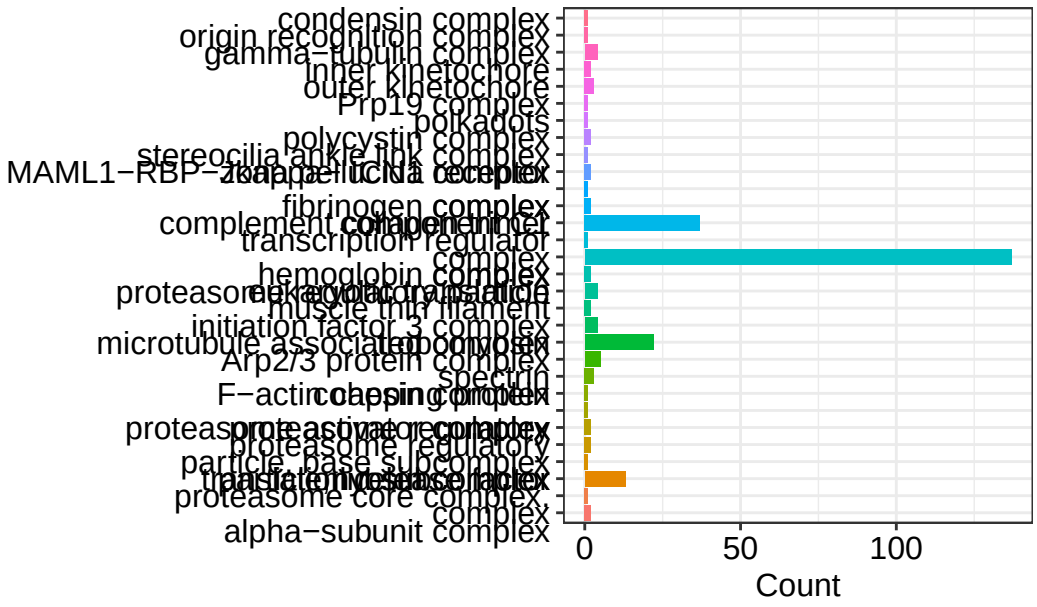
```
enrichplot v1.30.4 Learn more at https://yulab-smu.top/contribution-knowledge-
mining/

Please cite:

Guangchuang Yu, Li-Gen Wang, and Qing-Yu He. ChIPseeker: an
R/Bioconductor package for ChIP peak annotation, comparison and
visualization. Bioinformatics. 2015, 31(14):2382-2383
```

```
# BiocManager::install("ggnewscale")
library("ggnewscale")

OrgDb <- org.Hs.eg.db # can also be other organisms
# Get ENTREZID as vector. genes list are gonna be used to call GO terms
genes <- as.character(resSig$ENTREZID)


# GeneOntology terms

## Cellular compartment
# In clusterProfiler, groupGO is designed for gene classification based on GO distribution at
ggo <- clusterProfiler::groupGO(gene     = genes,
                                OrgDb    = OrgDb,
                                ont      = "CC",     #MF , BP CC
                                level    = 3,
                                readable = TRUE)
head(as.data.frame(ggo)[,-5])
```

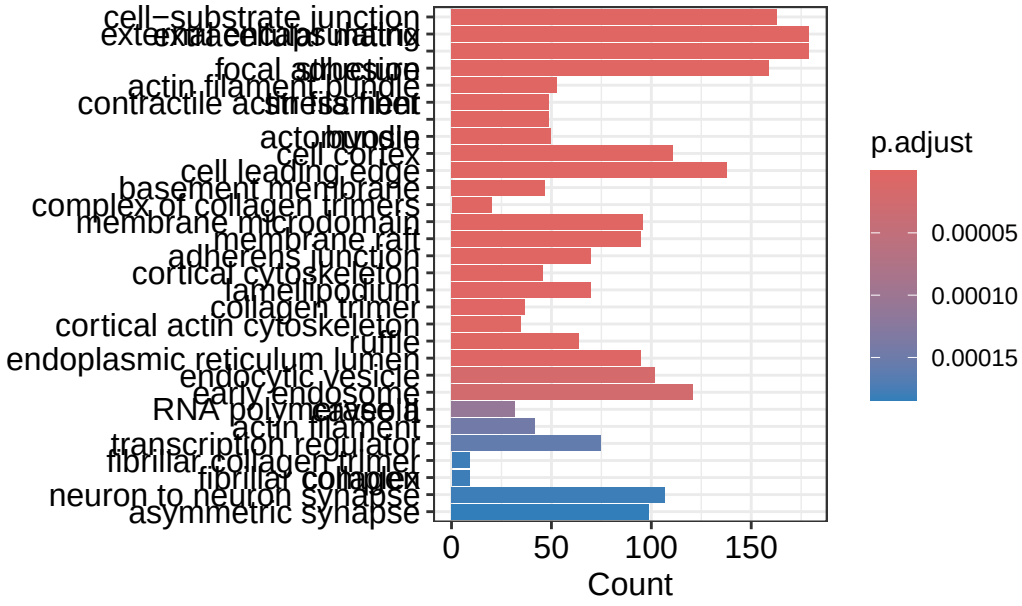
```
                   ID                Description Count GeneRatio
GO:0000133 GO:0000133                 polarisome     0    0/3777
GO:0000417 GO:0000417                HIR complex     0    0/3777
```

```
GO:0000796 GO:0000796          condensin complex     1    1/3777
GO:0000808 GO:0000808 origin recognition complex     1    1/3777
GO:0000930 GO:0000930      gamma-tubulin complex     4    4/3777
GO:0000939 GO:0000939           inner kinetochore     2    2/3777
```

```r
barplot(ggo, drop=TRUE, showCategory=30, vertex.label.cex=0.8)
```




```r
# GO over-representation test
ego <- clusterProfiler::enrichGO(gene          = genes,
                                 OrgDb         = OrgDb,
                                 ont           = "CC",
                                 pAdjustMethod = "BH",
                                 pvalueCutoff  = 0.01,
                                 qvalueCutoff  = 0.01,
                                 readable      = TRUE)


barplot(ego, showCategory=30)
```




```
# Gene Concept Network
edox <- setReadable(ego, 'org.Hs.eg.db', 'ENTREZID')

## Gene list with Fold Change data
geneList <- resSig$log2FoldChange
names(geneList) <- as.character(unique(resSig$ENTREZID))
geneList <- sort(geneList, decreasing = TRUE)
#de <- names(geneList)[abs(geneList) > 2]
head(geneList)
```

```
   23321     7704       59    26135    84909    54456
9.505982 7.352645 6.326295 5.884912 5.207201 5.090427
```

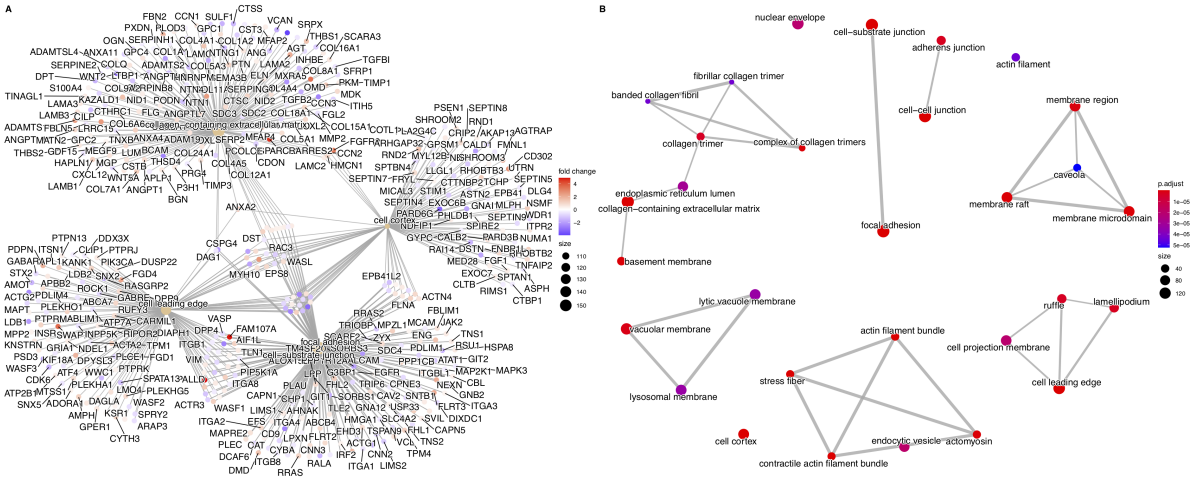
```
## categorySize can be scaled by 'pvalue' or 'geneNum'
# p1 <- cnetplot(edox, categorySize="geneNum", foldChange=geneList)
p1 <- cnetplot(edox, size_category=0.5, foldChange=geneList)

# Enrichment Map
# Enrichment map organizes enriched terms into a network with edges connecting overlapping ge
# In this way, mutually overlapping gene sets are tend to cluster together, making it easy to
library(DOSE)
```

```
DOSE v4.4.0 Learn more at https://yulab-smu.top/contribution-knowledge-
mining/

Please cite:

Guangchuang Yu, Li-Gen Wang, Guang-Rong Yan, Qing-Yu He. DOSE: an
R/Bioconductor package for Disease Ontology Semantic and Enrichment
analysis. Bioinformatics. 2015, 31(4):608-609
```

```r
edox <- pairwise_termsim(edox)
p2 <- emapplot(edox, layout="kk")

# Plot Gene Concept Network and Enrichment Map
 pdf(file=paste("Merge_GO_Gene_Set_Enrichment_Analysis", "CC", ".pdf", sep="_"),
      width=25, height=10)
cowplot::plot_grid(p1, p2, ncol=2, labels=LETTERS[1:3], rel_widths=c(.8, .8, 1.2))
dev.off()
```

```
pdf
  2
```



References

Anders, Simon, and Wolfgang Huber. 2010. “Differential expression analysis for sequence count data.” Genome Biology 11 (10):R106+. https://doi.org/10.1186/gb-2010-11-10-r106.

Dudoit, Rine, Yee H. Yang, Matthew J. Callow, and Terence P. Speed. 2002. "Statistical methods for identifying differentially expressed genes in replicated cDNA microarray experiments." Statistica Sinica, 111–39.

Love, Michael I., Wolfgang Huber, and Simon Anders. 2014. "Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2." Genome Biology 15 (12). BioMed Central Ltd:550+. https://doi.org/10.1186/s13059-014-0550-8.

Michael Love. RNA-seq workflow: gene-level exploratory analysis and differential expression. 2018 **
https://www.bioconductor.org/packages/devel/workflows/vignettes/rnaseqGene/inst/doc/rnaseqGene.html

Himes, Blanca E., Xiaofeng Jiang, Peter Wagner, Ruoxi Hu, Qiyu Wang, Barbara Klanderman, Reid M. Whitaker, et al. 2014. "RNA-Seq transcriptome profiling identifies CRISPLD2 as a glucocorticoid responsive gene that modulates cytokine function in airway smooth muscle cells." PloS One 9 (6). https://doi.org/10.1371/journal.pone.0099625.

Session Info

We should always keep record of the R packages used and their versions. In some cases you could get different results with different packages version!!

```
devtools::session_info()
```

```
- Session info -------------------------------------------------------------
 setting  value
 version  R version 4.5.2 (2025-10-31)
 os       Ubuntu 22.04.5 LTS
 system   x86_64, linux-gnu
 ui       X11
 language (EN)
 collate  en_US.UTF-8
 ctype    en_US.UTF-8
 tz       Etc/UTC
 date     2026-02-25
 pandoc   3.6.3 @ /usr/lib/rstudio-server/bin/quarto/bin/tools/x86_64/ (via rmarkdown)
 quarto   1.8.25 @ /usr/lib/rstudio-server/bin/quarto/bin/quarto

- Packages -----------------------------------------------------------------
 package              * version    date (UTC) lib source
 abind                  1.4-8      2024-09-12 [1] RSPM (R 4.5.2)
 affy                   1.88.0     2025-10-29 [1] Bioconductor 3.22 (R 4.5.2)
```

```
affyio                1.80.0     2025-10-29 [1] Bioconductor 3.22 (R 4.5.2)
airway              * 1.30.0     2025-10-30 [1] Bioconductor 3.22 (R 4.5.2)
AnnotationDbi       * 1.72.0     2025-10-29 [1] Bioconductor 3.22 (R 4.5.2)
ape                   5.8-1      2024-12-16 [1] RSPM (R 4.5.2)
apeglm              * 1.32.0     2025-10-29 [1] Bioconductor 3.22 (R 4.5.2)
aplot                 0.2.9      2025-09-12 [1] RSPM (R 4.5.2)
bbmle                 1.0.25.1   2023-12-09 [1] RSPM (R 4.5.2)
bdsmatrix             1.3-7      2024-03-02 [1] RSPM (R 4.5.2)
beeswarm              0.4.0      2021-06-01 [1] CRAN (R 4.5.2)
Biobase             * 2.70.0     2025-10-29 [1] Bioconductor 3.22 (R 4.5.2)
BiocGenerics        * 0.56.0     2025-10-29 [1] Bioconductor 3.22 (R 4.5.2)
BiocManager           1.30.27    2025-11-14 [1] RSPM (R 4.5.2)
BiocParallel          1.44.0     2025-10-29 [1] Bioconductor 3.22 (R 4.5.2)
Biostrings            2.78.0     2025-10-29 [1] Bioconductor 3.22 (R 4.5.2)
bit                   4.6.0      2025-03-06 [1] CRAN (R 4.5.2)
bit64                 4.6.0-1    2025-01-16 [1] CRAN (R 4.5.2)
blob                  1.3.0      2026-01-14 [1] CRAN (R 4.5.2)
cachem                1.1.0      2024-05-16 [1] CRAN (R 4.5.2)
cli                   3.6.5      2025-04-23 [1] CRAN (R 4.5.2)
cluster               2.1.8.2    2026-02-05 [2] CRAN (R 4.5.2)
clusterProfiler     * 4.18.4     2025-12-15 [1] Bioconductor 3.22 (R 4.5.2)
coda                  0.19-4.1   2024-01-31 [1] RSPM (R 4.5.2)
codetools             0.2-20     2024-03-31 [1] CRAN (R 4.5.2)
cowplot               1.2.0      2025-07-07 [1] RSPM (R 4.5.2)
crayon                1.5.3      2024-06-20 [1] CRAN (R 4.5.2)
data.table            1.18.2.1   2026-01-27 [1] CRAN (R 4.5.2)
DBI                   1.3.0      2026-02-25 [1] CRAN (R 4.5.2)
DelayedArray          0.36.0     2025-10-29 [1] Bioconductor 3.22 (R 4.5.2)
DESeq2              * 1.50.2     2025-11-13 [1] Bioconductor 3.22 (R 4.5.2)
devtools              2.4.6      2025-10-03 [1] CRAN (R 4.5.2)
digest                0.6.39     2025-11-19 [1] CRAN (R 4.5.2)
DOSE                * 4.4.0      2025-10-29 [1] Bioconductor 3.22 (R 4.5.2)
dplyr               * 1.2.0      2026-02-03 [1] CRAN (R 4.5.2)
ellipsis              0.3.2      2021-04-29 [1] CRAN (R 4.5.2)
emdbook               1.3.14     2025-07-23 [1] RSPM (R 4.5.2)
enrichplot          * 1.30.4     2025-12-01 [1] Bioconductor 3.22 (R 4.5.2)
evaluate              1.0.5      2025-08-27 [1] CRAN (R 4.5.2)
farver                2.1.2      2024-05-13 [1] CRAN (R 4.5.2)
fastmap               1.2.0      2024-05-15 [1] CRAN (R 4.5.2)
fastmatch             1.1-8      2026-01-17 [1] RSPM (R 4.5.2)
fgsea                 1.36.2     2026-01-05 [1] Bioconductor 3.22 (R 4.5.2)
fontBitstreamVera     0.1.1      2017-02-01 [1] RSPM (R 4.5.2)
fontLiberation        0.1.0      2016-10-15 [1] RSPM (R 4.5.2)
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fontquiver             0.2.1      2017-02-01 [1] RSPM (R 4.5.2)
fs                     1.6.6      2025-04-12 [1] CRAN (R 4.5.2)
gdtools                0.5.0      2026-02-09 [1] RSPM (R 4.5.2)
generics             * 0.1.4      2025-05-09 [1] CRAN (R 4.5.2)
GenomeInfoDb           1.46.2     2025-12-04 [1] Bioconductor 3.22 (R 4.5.2)
GenomicRanges        * 1.62.1     2025-12-08 [1] Bioconductor 3.22 (R 4.5.2)
ggbeeswarm           * 0.7.3      2025-11-29 [1] CRAN (R 4.5.2)
ggforce                0.5.0      2025-06-18 [1] RSPM (R 4.5.2)
ggfun                  0.2.0      2025-07-15 [1] RSPM (R 4.5.2)
ggiraph                0.9.6      2026-02-21 [1] RSPM (R 4.5.2)
ggnewscale           * 0.5.2      2025-06-20 [1] CRAN (R 4.5.2)
ggplot2              * 4.0.2      2026-02-03 [1] CRAN (R 4.5.2)
ggplotify              0.1.3      2025-09-20 [1] RSPM (R 4.5.2)
ggrepel              * 0.9.7      2026-02-25 [1] CRAN (R 4.5.2)
ggtangle               0.1.1      2026-01-16 [1] RSPM (R 4.5.2)
ggtree                 4.0.4      2026-01-05 [1] Bioconductor 3.22 (R 4.5.2)
glue                   1.8.0      2024-09-30 [1] CRAN (R 4.5.2)
GO.db                  3.22.0     2026-02-25 [1] Bioconductor
GOSemSim               2.36.0     2025-10-29 [1] Bioconductor 3.22 (R 4.5.2)
gridGraphics           0.5-1      2020-12-13 [1] RSPM (R 4.5.2)
gson                   0.1.0      2023-03-07 [1] RSPM (R 4.5.2)
gtable                 0.3.6      2024-10-25 [1] CRAN (R 4.5.2)
hexbin               * 1.28.5     2024-11-13 [1] CRAN (R 4.5.2)
htmltools              0.5.9      2025-12-04 [1] CRAN (R 4.5.2)
htmlwidgets            1.6.4      2023-12-06 [1] RSPM (R 4.5.2)
httr                   1.4.8      2026-02-13 [1] CRAN (R 4.5.2)
igraph                 2.2.2      2026-02-12 [1] RSPM (R 4.5.2)
IRanges              * 2.44.0     2025-10-29 [1] Bioconductor 3.22 (R 4.5.2)
jsonlite               2.0.0      2025-03-27 [1] CRAN (R 4.5.2)
KEGGREST               1.50.0     2025-10-29 [1] Bioconductor 3.22 (R 4.5.2)
knitr                  1.51       2025-12-20 [1] CRAN (R 4.5.2)
labeling               0.4.3      2023-08-29 [1] CRAN (R 4.5.2)
lattice                0.22-9     2026-02-09 [1] CRAN (R 4.5.2)
lazyeval               0.2.2      2019-03-15 [1] CRAN (R 4.5.2)
lifecycle              1.0.5      2026-01-08 [1] CRAN (R 4.5.2)
limma                  3.66.0     2025-10-29 [1] Bioconductor 3.22 (R 4.5.2)
locfit                 1.5-9.12   2025-03-05 [1] RSPM (R 4.5.2)
magrittr               2.0.4      2025-09-12 [1] CRAN (R 4.5.2)
MASS                   7.3-65     2025-02-28 [2] RSPM (R 4.4.0)
Matrix                 1.7-4      2025-08-28 [2] RSPM (R 4.5.0)
MatrixGenerics       * 1.22.0     2025-10-29 [1] Bioconductor 3.22 (R 4.5.2)
matrixStats          * 1.5.0      2025-01-07 [1] RSPM (R 4.5.2)
memoise                2.0.1      2021-11-26 [1] CRAN (R 4.5.2)
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mvtnorm                1.3-3      2025-01-10 [1] RSPM (R 4.5.2)
nlme                   3.1-168    2025-03-31 [2] RSPM (R 4.4.0)
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org.Hs.eg.db         * 3.22.0     2026-02-25 [1] Bioconductor
otel                   0.2.0      2025-08-29 [1] CRAN (R 4.5.2)
patchwork              1.3.2      2025-08-25 [1] CRAN (R 4.5.2)
pheatmap             * 1.0.13     2025-06-05 [1] CRAN (R 4.5.2)
pillar                 1.11.1     2025-09-17 [1] CRAN (R 4.5.2)
pkgbuild               1.4.8      2025-05-26 [1] CRAN (R 4.5.2)
pkgconfig              2.0.3      2019-09-22 [1] CRAN (R 4.5.2)
pkgload                1.5.0      2026-02-03 [1] CRAN (R 4.5.2)
plyr                   1.8.9      2023-10-02 [1] RSPM (R 4.5.2)
png                    0.1-8      2022-11-29 [1] RSPM (R 4.5.2)
polyclip               1.10-7     2024-07-23 [1] RSPM (R 4.5.2)
preprocessCore         1.72.0     2025-10-29 [1] Bioconductor 3.22 (R 4.5.2)
purrr                  1.2.1      2026-01-09 [1] CRAN (R 4.5.2)
qvalue                 2.42.0     2025-10-29 [1] Bioconductor 3.22 (R 4.5.2)
R.methodsS3            1.8.2      2022-06-13 [1] CRAN (R 4.5.2)
R.oo                   1.27.1     2025-05-02 [1] CRAN (R 4.5.2)
R.utils                2.13.0     2025-02-24 [1] CRAN (R 4.5.2)
R6                     2.6.1      2025-02-15 [1] CRAN (R 4.5.2)
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Rcpp                   1.1.1      2026-01-10 [1] CRAN (R 4.5.2)
remotes                2.5.0      2024-03-17 [1] CRAN (R 4.5.2)
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rlang                  1.1.7      2026-01-09 [1] CRAN (R 4.5.2)
rmarkdown              2.30       2025-09-28 [1] CRAN (R 4.5.2)
RSQLite                2.4.6      2026-02-06 [1] RSPM (R 4.5.2)
rstudioapi             0.18.0     2026-01-16 [1] CRAN (R 4.5.2)
S4Arrays               1.10.1     2025-12-01 [1] Bioconductor 3.22 (R 4.5.2)
S4Vectors            * 0.48.0     2025-10-29 [1] Bioconductor 3.22 (R 4.5.2)
S7                     0.2.1      2025-11-14 [1] CRAN (R 4.5.2)
scales                 1.4.0      2025-04-24 [1] CRAN (R 4.5.2)
scatterpie             0.2.6      2025-09-12 [1] RSPM (R 4.5.2)
Seqinfo              * 1.0.0      2025-10-29 [1] Bioconductor 3.22 (R 4.5.2)
sessioninfo            1.2.3      2025-02-05 [1] CRAN (R 4.5.2)
SparseArray            1.10.8     2025-12-18 [1] Bioconductor 3.22 (R 4.5.2)
statmod                1.5.1      2025-10-09 [1] RSPM (R 4.5.2)
stringi                1.8.7      2025-03-27 [1] CRAN (R 4.5.2)
stringr                1.6.0      2025-11-04 [1] CRAN (R 4.5.2)
SummarizedExperiment * 1.40.0     2025-10-29 [1] Bioconductor 3.22 (R 4.5.2)
systemfonts            1.3.1      2025-10-01 [1] CRAN (R 4.5.2)
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 treeio                1.34.0     2025-10-30 [1] Bioconductor 3.22 (R 4.5.2)
 tweenr                2.0.3      2024-02-26 [1] RSPM (R 4.5.2)
 UCSC.utils            1.6.1      2025-12-11 [1] Bioconductor 3.22 (R 4.5.2)
 usethis               3.2.1      2025-09-06 [1] CRAN (R 4.5.2)
 vctrs                 0.7.1      2026-01-23 [1] CRAN (R 4.5.2)
 vipor                 0.4.7      2023-12-18 [1] CRAN (R 4.5.2)
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 withr                 3.0.2      2024-10-28 [1] CRAN (R 4.5.2)
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 XVector               0.50.0     2025-10-29 [1] Bioconductor 3.22 (R 4.5.2)
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 [1] /usr/local/lib/R/site-library
 [2] /usr/lib/R/site-library
 [3] /usr/lib/R/library
 * -- Packages attached to the search path.

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